# Molecular Mechanisms of Insecticide Resistance

## Diversity Among Insects

EDITED BY
Christopher A. Mullin
and Jeffrey G. Scott

ACS Symposium Series [illegible]

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ACS SYMPOSIUM SERIES 505

# Molecular Mechanisms of Insecticide Resistance

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# Foreword

THE ACS SYMPOSIUM SERIES was first published in 1974 to provide a mechanism for publishing symposia quickly in book form. The purpose of this series is to publish comprehensive books developed from symposia, which are usually "snapshots in time" of the current research being done on a topic, plus some review material on the topic. For this reason, it is necessary that the papers be published as quickly as possible.

Before a symposium-based book is put under contract, the proposed table of contents is reviewed for appropriateness to the topic and for comprehensiveness of the collection. Some papers are excluded at this point, and others are added to round out the scope of the volume. In addition, a draft of each paper is peer-reviewed prior to final acceptance or rejection. This anonymous review process is supervised by the organizer(s) of the symposium, who become the editor(s) of the book. The authors then revise their papers according the the recommendations of both the reviewers and the editors, prepare camera-ready copy, and submit the final papers to the editors, who check that all necessary revisions have been made.

As a rule, only original research papers and original review papers are included in the volumes. Verbatim reproductions of previously published papers are not accepted.

*M. Joan Comstock*
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# Preface

INSECT AND MITE RESISTANCE TO INSECTICIDES/ACARICIDES, documented since 1908, is found in more than 500 species and globally is the major obstacle to dependable control of agricultural and medical pests. Virtually every chemical and microbial agent is prone to resistance development in arthropods. Effects of resistance include reduced crop yields and the need for more frequent pesticide applications or higher pesticide dosages, which can result in environmental damage. Resistance can also result in outbreaks of arthropod-borne human and veterinary diseases. Resistances within or between whole classes of insecticides, including new control agents in commercial development, are an ever-increasing problem for control of major crop pests. Given the tremendous difficulty and investment associated with the development of new insecticides that are safe and cost-effective, there is a grave need to preserve the efficacy of current and future insecticides. Thus, we need to understand the mechanisms by which insects acquire resistance so that we can intelligently design strategies to delay its onset.

Recently, the techniques of molecular biology have come to bear on the problem of understanding the basis of insecticide resistance. We are indeed fortunate to have available molecular tools that isolate rare genes out of complex mixtures of nucleic acids. These techniques have been expanded to many agriculturally and medically important pests. Classically, three major resistance mechanisms to toxic chemicals have been identified in insects: increased detoxification, insensitive target sites, and decreased penetration. A combination of factors can lead to greatly potentiated resistance levels. Classification of these mechanisms can now be in molecular terms; gene amplification and changes in structural genes or in gene expression are the best documented bases for insecticide resistance in insects.

Presented in this volume are recent advances by experts from around the globe on the molecular basis of insecticide resistance. Our goal is to present major breakthroughs concerning not only houseflies and fruit flies of the Diptera, but also the phytophagous members of major insect pest orders including Lepidoptera, Coleoptera, Homoptera, and Orthoptera. Some emphasis has been given to the herbivore model and its relevance to biochemical and genetic strategies that confer resistance to natural, synthetic, or bioengineered insecticides. This volume should assist toxicologists, pharmacologists, chemical ecologists, endocrinologists, molecu-

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lar biologists, environmental chemists, and regulatory scientists in their labors with pesticides and other toxic substances.

It is easy to get caught up in the marvel of resistance—the fact that a given strain can survive several thousandfold more toxicant than a "normal" strain—and the amazing biochemical and genetic traits that allow for this survival. In this book we have tried to present an overview of all these different areas, including involvement of cytochrome P450s, glutathione transferases, carboxylesterases, and other hydrolases as well as altered acetylcholinesterases, sodium channels, $\gamma$-aminobutyric acid ionophore complexes, and *Bacillus thuringiensis* toxin and juvenile hormone receptors. However, many gaps in knowledge persist. For example, resistance mechanisms to insecticides/acaricides have been characterized in only a few beneficial arthropods, and they have been only preliminarily addressed at the molecular level. The transfer of technologies among investigators working in different areas, including the transfer of gene probes between investigators working on model systems such as *Drosophila* and those working on agriculturally and medically important insects and Acari, should be stimulated by the work presented here. Only through this type of communication can the field as a whole go forward.

New methods for selecting, detecting, cloning, purifying, and expressing resistance genes and for characterizing the real-time and -space dynamics of insecticide interactions with resistance gene products will be needed to ensure future advances in resolving the full diversity of resistance mechanisms available to insects. This information will provide the rationale for both prolonging the lifetime of a pesticide and for the appropriate use of insecticide mixtures with synergists or control agents with multiple, selective sites of action to delay resistance development. Future implementation of transgenic or conventional insecticides with lasting utility will depend on our knowledge of the molecular dynamics of insecticide resistance.

We would like to acknowledge our expert authors for their rich and timely contributions and our colleagues for their critical reviews. Their efforts and the generous financial support of the Agrochemical Division of the American Chemical Society and the Insecticide Resistance Action Committee made this volume possible.

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# Insecticide Resistance Action Committee

With the objective of prolonging the effectiveness of insecticides by countering resistance problems, the International Group of National Associations of Agrochemical Manufacturers (GIFAP) formed the Insecticide Resistance Action Committee (IRAC) in 1984. Its membership, drawn from Europe, the United States, and Japan, comprises senior technical people from agrochemical companies affiliated with GIFAP through membership of the relevant national association.

IRAC has the following major functions:

- to provide expert advice to GIFAP on technical matters relating to insecticide resistance and provide support in representing the industry view on insecticide resistance;
- to establish relationships with nonindustry researchers in the field of insecticide resistance by means of joint seminars, conferences, and research projects; and
- to coordinate industry efforts to prolong the life of insecticides and acaricides in the face of resistance by defining and recommending appropriate strategies.

To carry out these functions, working groups have been established for each major crop or outlet where resistance problems occur. These working groups are the Cotton, Stored Products, Rice, Public Health and Vectors, Fruit Crops, Ectoparasites, and Field Crops/Vegetables working groups, and the Pyrethroid and the *Bacillus thuringiensis* efficacy groups.

Partial support for the ACS symposium on which this book is based was provided by IRAC. Reports of IRAC's activities are published in the *FRAC/IRAC Newsletter*, issued with the *GIFAP Bulletin*. Further information can be obtained from the IRAC Communications Officer, R. W. Lemon, Schering Agrochemicals Limited, Chesterford Park Research Station, Saffron Walden, Essex CB10 1XL, United Kingdom.

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## Chapter 1

# Biomolecular Basis for Insecticide Resistance

## Classification and Comparisons

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Insecticide resistance, documented in nearly all arthropod species in which it has been studied, is a major obstacle to control of agriculturally and medically important pests. This book presents recent major breakthroughs in understanding, at the molecular level, the mechanisms by which insects acquire resistance to natural, synthetic or bioengineered insecticides. This chapter will summarize these advances, especially as they relate to the classification and comparative aspects of resistance mechanisms. Biochemodiversity, particularly in cytochrome P450s, is evident for resistance development among different insect species. Multiresistance involving target sites can be overcome with novel-acting chemistry or insecticides that do not allosterically interact with insensitive domains. Future implementation of transgenically or exogenously introduced insecticides will depend on our knowledge of the molecular dynamics of insecticide resistance.

### The Problem of Insecticide Resistance

Insecticide resistance is the major obstacle to control of agriculturally and medically important pests. This worldwide problem has been documented for over 500 arthropod species, particularly among flies, caterpillars, beetles and mites (*1*). Resistance results in increased pesticide application frequencies, increased dosages, decreased yields, environmental damage and outbreaks of arthropod-borne human and veterinary diseases. Resistances within or between whole classes of insecticides are an ever increasing problem for control of major crop pests. Given the tremendous difficulty and investment associated with development of new, safe and cost-effective insecticides (2), there is a grave need to preserve the efficacy of current and future insecticides. For these reasons, it is essential to understand the mechanisms by which insects acquire resistance so that we can intelligently design strategies to delay its onset.

**What Is Resistance?** The World Health Organization defines *resistance* as "the development of an ability in a strain of an organism to tolerate doses of a toxicant which would prove lethal to the majority of individuals in a normal (susceptible) population of the species" (*3*). Implied is the inheritability of this characteristic or trait, and as such can be used to identify a certain population of a species. Indeed, the more one knows about the repertoire of chemistry that an insect is resistant or susceptible to and the molecular mechanisms underlying these traits, the more chemotaxonomically identified

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the population becomes. Similar traits (e.g. antibiotic or drug sensitivity and its associated resistance mechanism; kinds of non-autotoxic poisons produced) can molecularly classify a microbe (*4, 5*).

*Resistance development* in insects to acutely toxic insecticides generally occurs by selection of rare individuals in a population that can survive the insecticide; it is pre-adaptive and not a mutational effect (*6-9*). Most commercial insecticides are for safety reasons designed to be poor mutagens, and their use results in an intense chemical selection (high dose, high lethality) which is not conducive to genetic alterations but allows survival of pre-adapted, i. e. resistant individuals (*10*). Individuals predisposed for resistance to insecticides never before encountered originate from the complex interplay between normally occurring genetic errors, as well as naturally occurring or synthetic genotoxins (e.g. dietary and environmental mutagens).

Herein lies a general problem in use of resistance terminology among insecticide toxicologists investigating lab strains selected with a high dose of a highly toxic synthetic compared to ecological biochemists studying field populations undergoing "coevolution" with less acutely toxic secondary plant chemicals. Long term toxicant-insect interactions in a naturally mutagenic environment may indeed select for advantageous mutations, and allelochemically-induced resistance (also known as tolerance) can occur. Within generation induction of "resistance" particularly through detoxifying enzymes and inheritable pre-adapted resistances to natural insecticides become converging concepts, and the classical terms (*6-8*) of *tolerance* (decreased susceptibility within lifetime of insect) and *resistance* (heritable) tend to merge. With this in mind, there will be some variability throughout this book in how these terms are used. Nevertheless, *cross-resistance* (resistance to one compound that confers by a common mechanism resistance to another xenobiotic) and *multiple or multiresistance* (resistance to more than one or a class of compounds due to coexistence of separate mechanisms) are more uniformly used in this volume.

**A Brief History.** The first documented case of insecticide resistance in arthropods was 1908 in Washington for the San Jose scale *Quadraspidiotus perniciosus* to lime-sulfur (cited in *11*). Incidence of resistance in the "field" has generally correlated with the length of time an insecticide has been used, hence the trend among insecticide classes is organochlorines > organophosphates > carbamates > pyrethroids > insect growth regulators, microbials etc. in number of documented cases (*1, 12*). Resistances to bacterial pesticides (*13*) and to baculoviruses (*14*) up to 800-fold have already been noted in insect populations. Virtually every chemical and microbial agent is prone to resistance development in arthropods. Often a lack of exposure is involved in species slow to exhibit resistance (cotton boll weevil feeding within boll; European corn borer feeding within corn stalk) rather than some intrinsic propensity for susceptibility (*15*). Entrenched multiple resistance is becoming a major problem for success of new agrochemicals. Colorado potato beetle, *Leptinotarsa decemlineata*, is a case in point where it has developed so many resistances that its control is no longer assured with novel insecticides. Best demonstrated at the molecular level in house fly, *Musca domestica* (see below), mechanisms in these multiresistant pests may be converging to the extent that at least one population has some cross-resistance to new control agents in commercial development. Chemical combination with synergists to retard resistance-conferring enzymes, while not necessarily successful with the Colorado potato beetle (*16*), has been one strategy used to delay resistance with these problematic pests.

## The Molecular Age

Even as late as five years ago, Dr. F. J. Oppenoorth concluded that after thirty years of intensive study the molecular genetics of any case of insecticide resistance in house fly was still not understood (*17*). We are indeed fortunate today to have ready available molecular tools to pull relatively rare genes out of what was formerly considered to be a hopeless *pot pouri* of nucleic acids. The classification of resistance mechanisms has gone molecular; determining homology and amino acid identity are now evolving towards the need to define the three-dimensional architecture that provides the right active space or conformation for the critical chemical interaction with an enzyme or receptor to take place. We now have the technology to elucidate chemical resistance mechanisms in the least of tissues with the most discrete of biospecific functions. Genetic expression systems such as baculovirus recombinants introduced into insect cell lines and *Xenopus* oocytes are now available to render functional the products of foreign nucleic acids. These are exciting times!

Over the past several years, the techniques of molecular biology have come to bear on the problem of understanding the basis of insecticide resistance. These techniques have generally been pioneered in house flies and fruit flies, and have not been expanded to many agriculturally important pests. Our goal in this book is to present recent advances by experts from around the globe on the molecular basis of insecticide resistance, focussing, in addition to genetically mapped species, on the herbivorous members of major insect orders including Diptera, Lepidoptera, Coleoptera, Homoptera and Orthoptera. Biochemical and genetic strategies that confer resistance to synthetic and natural insect control molecules will be presented, with some emphasis on the herbivore model. This effort will update and supplement other works, most notably that of Georghiou and Saito (*18*), Committee on Strategies ... (*19*), Ford et al. (*20*), Green et al.(*21*), and Roush and Tabashnik (22), and provide rationale for future development of successful pest control agents with lasting utility.

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## Molecular Classification of Insecticide Resistance

For many years it has been known that insects could become resistant *behaviorally* (avoid exposure to a lethal dose) or *physiologically* (find ways to survive a normally lethal dose) to insecticides. Behavioral mechanisms for resistance (*23*) have been much less studied than physiological, even though avoidance of non-host chemicals is a major route by which insects select foods. Physiological resistance has been divided into mechanisms of reduced cuticular penetration, altered target site, increased metabolic detoxication (or decreased metabolic activation, e.g. phosphorothionate) or sequestration (*9*, *24*, *25*). Increased excretion (i.e. facilitated transport of parent insecticide; not just the consequence of more passively excretable metabolites) is also a possible resistance mechanism, although it has never been convincingly demonstrated to our knowledge. Often a combination of factors is involved which greatly potentiates the overall resistance level.

Such an empirical explanation of resistance mechanisms is no longer satisfactory to biochemists and may never have been of much use to geneticists. A mechanism, whether physiological or behavioral, has a more fundamental biochemical and ultimately a molecular (DNA) basis. Identifying the responsible nucleic acid sequence and its functional role in resistance is a more foundational understanding than the correlation of enzyme activities with resistance; the latter could result from linked expression with the authentic resistance-conferring gene. In turn, the same R-gene may evoke co-occurring behavioral and physiological resistances to antifeedant, hormonal, anti-growth, and neurotoxic effects etc. of a specific or related chemical depending on the tissue or cell site of its expression. With the tools of molecular biology we are now poised on the verge of a new era, one in which our understanding of the molecular mechanisms of resistance, both in terms of its genetic control and biochemical regulation, will vastly improve. A molecular classification initially developed by Carino and Feyereisen (*26*) is modified here (Table I) as a general summary of what is currently known at the gene level about physiological and behavioral mechanisms of insecticide resistance (cf *27*).

Table I. Molecular Classification of Insect Resistance Mechanisms[a]

| *Resistance Mechanism* | *Change in Structural Gene* | *Gene Amplification* | *Change in Gene Expression* |
|---|---|---|---|
| Increased Metabolism | | | |
| Esterase | ND[b] | + (OP, PYR[c]) | + (OP, PYR)[d] |
| P450 Monooxygenase | ND | ND | + (PYR) |
| Glutathione transferase | ? (OP) | ND | ? (OP) |
| Target Site Insensitivity | | | |
| Acetylcholinesterase | + (OP, CAR) | ND | ND |
| GABA receptor complex | ? (CYC) | ND | ND |
| Sodium channel | ? (DDT, PYR) | ND | ND |
| JH receptor | ? (JH, JHA) | ND | ND |
| BT receptor | ? (BTs) | ND | ND |
| Reduced Penetration | ND | ND | ND |
| Behavior | ND | ND | ND |

[a] Adapted from Carino and Feyereisen (*26*).

[b] ND = no data or limited negative data; ? = some supporting evidence; + = good supporting evidence.

[c] BT(s) = *Bacillus thuringiensis* toxins; CAR = carbamates; CYC = cyclodienes; JH, JHA = juvenile hormone and analogues; OP = organophosphates; PYR = pyrethroids; .

[d] Regulated by DNA methylation, demethylation (*28*).

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**Examples Based on Increased Metabolism.** An important mechanism to increase levels of resistance-conferring detoxification enzymes is gene amplification (*28*). This mechanism has been clearly demonstrated in aphids where the E4 and FE4 esterase genes, remarkably activated by transcriptional methylation of cytosine residues in the 3' region, are amplified to give multi-insecticide resistance to both the green peach aphid, *Myzus persicae*, and the related tobacco aphid, *M. nicotianae* (see chapter **17**). Reversion with release of insecticide pressure is associated with 5-demethylation of cytosine in the esterase gene. Amplification of esterase genes has also been identified with organophosphate resistance in the *Culex pipiens* complex (*29*).

Resistance can also occur by changes in gene expression other than amplification. For example, the level of specific P450 mRNAs (see chapters **2-4**) and glutathione transferase mRNAs (*30*, *31*; see chapter **5**) are known to be higher in resistant strains than susceptible strains, and a mechanism other than amplification is likely (see chapter **4**). For example, Waters et al. have cloned the *CYP6A2* gene from susceptible and resistant strains of *Drosophila* and have found that the susceptible strain contains a long terminal repeat (LTR) from a transposable element inserted between the P450 coding region and the downstream polyadenylation site. They propose that this results in an unstable mRNA that leads to the lower expression of *CYP6A2* in the susceptible strain (chapter **4**). Interestingly, *trans* acting factors responsible for regulation of P450 expression in *Drosophila* (*32*) and house fly (Liu, N.; Scott, J. G.; unpublished) are also likely to exist.

**An Exciting Future.** Clearly, we have a long way to go before our understanding of resistance mechanisms is complete (Figure 1). However, researchers have made excellent strides in resolving insecticide resistance in certain cases. The biochemical basis of esterase mediated resistance in aphids (*28*) and mosquitos (*29*) are perhaps the best examples, although many more cases are likely to follow in the near future. For

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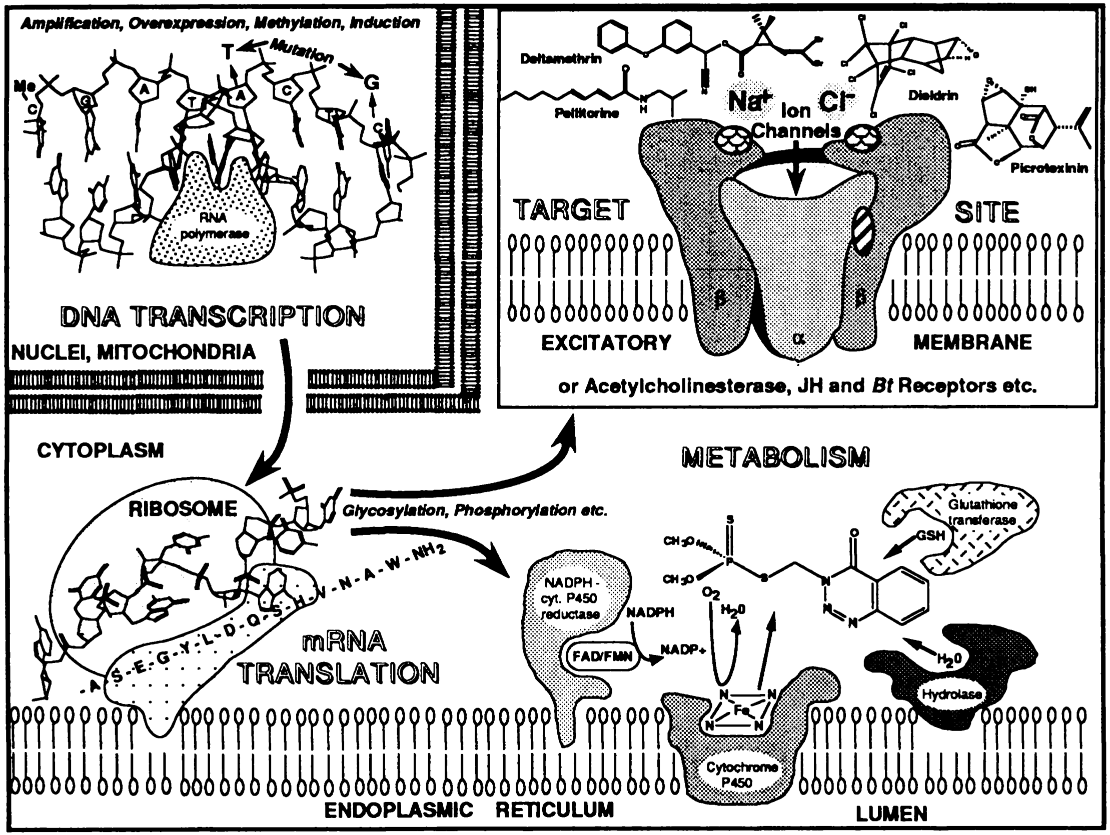
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H2O
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Fe
N
GSH
Glutathione transferase
H2O
Hydrolase
ENDOPLASMIC RETICULUM
LUMEN

Figure 1. Some major subcellular sites in the molecular interplays responsible for insecticide resistance. General features are presented with no attempt to resolve the discrete transcription, DNA processing, mRNA stabilization, translation and protein stabilization steps leading to proteins that confer resistance. The role of receptor (enzyme) - membrane interactions in functional resistance is unknown.

example, elucidation of the molecular basis of target site insensitivity in *kdr*, altered acetylcholinesterase, or altered juvenile hormone, picrotoxinin, *B. thuringiensis* or abamectin receptors is being realized by several different research groups (*33 -37*; see chapters **6-8**, **15**). It will be fascinating to learn the secrets of how much of a change is needed for an insect's target site to become insensitive. Is it a single base substitution? Is a change in several amino acids required, or is it possible that the association of the receptor with the membrane has somehow changed? The future of insecticide resistance studies looks very exciting indeed!

## New Techniques and Approaches to Understand Resistance in Insects

**Selection for Resistance Using Mutagenesis.** One of the interesting developments in the field of insecticide resistance has been in the methods used for selecting resistant strains. Although mutagenesis has been widely used by experimental biologists in the past, it has only recently been earnestly used to produce resistant strains of insects (see chapters **8**, **16**, **20**). Laboratory selections have proven to be good indicators of the genetics and biochemistry of resistance in field populations, although they require a proper pool of genetic diversity if the selections are to be meaningful. Whether the use of mutagens such as ethyl methanesulfonate will produce the same type of resistance (genetically and biochemically) as is found in the field remains an open question, but one certainly worthy of investigation.

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**Molecular Cloning and Purification of Resistance Gene Products.** For a thorough understanding of resistance mechanisms, it is clear that a variety of approaches will be needed. Two of the most important techniques are the successful cloning of the genes, and the purification of the proteins responsible for insecticide resistance. Only with the use of protein biochemistry and molecular genetics can the whole story of resistance become known. For example, expression of cytochrome P450s are known to be regulated at several steps in mammals including transcription, DNA processing, mRNA stabilization, translation and enzyme stabilization (*38*). Whether or not resistance can arise by similar mechanisms will only be determined through the use of antibodies, clones and their related technologies.

Based on studies presented in this book, it appears that there is no universal method for the cloning of insecticide resistance genes. Homology with vertebrate probes works sometimes, but not always. Examples of success in cloning resistance-related genes by screening expression libraries with antibodies, polymerase chain reactions (PCR) or brute force genetics (e.g. gene mapping, cosmid walk) in systems such as *Drosophila* are all found in this text.

While the principles of protein purification have not changed, recent advances, especially in HPLC, now allow for the purification of proteins that are relatively rare or come from limited amounts of starting materials. Microsequencing can then generate sufficient N- or C-terminus amino acid information to synthesize nucleic acid probes. Alternatively, enough protein at a high level of purity may be obtained for production of antibodies or direct characterization of the resistance-associated biomolecule.

**Expression Systems.** Another technique that will become of increasing importance is the use of expression systems. Whether the system is *Xenopus* oocytes to examine the neurophysiology of target site resistance genes (*39*) or insect cell-expressed baculovirus recombinants (*40*) for elucidating the substrate specificity of metabolic resistance genes, these systems will play an important role in insecticide resistance studies in the future. Expression systems may also allow for production of high levels of resistance-conferring proteins from a cloned gene. Such an enriched source of protein will greatly facilitate efforts to functionally characterize the mechanism responsible for resistance.

**Quantitative Structure Activity Relationships (QSAR).** Perhaps one of the strongest techniques to determine structure-function relationships relative to three-dimensional spatial molecular interactions with, for example, complex membrane ionophores is QSAR and molecular modelling software now available for personal computer use. Three-dimensional docking of insecticidal molecules to binding sites on receptor complexes or suitable templates representative of these domains can now be achieved. Software is available to estimate partial electrostatic charges, key torsion angles and to test in 3-D space goodness of fit to putative receptors (see chapter **23**). Multivariate QSAR equations using more specific hydrophobic, electronic and steric molecular descriptors in addition to model free-energy parameters compiled by Hansch and Leo (*41*) have been used successfully to characterize and optimize natural and synthetic insecticide-target interactions (*42*,*43*). This approach needs to be extended into studies addressing molecular resistances.

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## Comparison of Resistance Mechanisms Among Insect Species

**Is One Gene as Good as the Other?** All insects are not equal! Although obvious to most entomologists, this fact is not as conclusive to some molecular biologists who consider the *Drosophila* gene bank as the last word on insect resistance strategies. Diptera, most importantly *Drosophila melanogaster* and *Musca domestica*, remain the most genetically resolved of the insect species with well-characterized morphological markers linked to chromosome sites, and thus the species best suited to study inheritance and map genes linked to resistance (*44*). Other species such as *Tribolium castaneum* (see chapter **16**), the *Culex pipiens* complex (*29*), *Blattella germanica* (see chapter **18**), and more recently *Heliothis virescens* (*45*) are emerging as useful insect models for chromosome mapping, but these still inadequately represent the vast array of natural toxicant-gene interactions that undoubtedly have fostered "pre-adapted" strategies that allow herbivorous, carnivorous, hemophagous, caprophagous etc. insect pests to resist much of the largely synthetic arsenal of insecticides available to man. These natural encounters may be quite discrete such as a fungal endophyte within its host plant eliciting antifeedant or neurotoxic alkaloids that deter most herbivorous predators except for generalists such as the southern armyworm, *Spodoptera eridania*, endowed with appropriate alkaloid-metabolizing P450s (see chapter **10**).

**Biodiversity of Cytochrome P450s.** Greatly enhanced levels of a constitutively-expressed cytochrome $P450_{lpr}$ have been associated with pyrethroid resistance in the house fly (see chapter **2**). While immunoreactivity to a P450 in other house fly strains, including populations selectively resistant to alternative insecticide classes, has been found, this pyrethroid-metabolizing form has remarkably not been detected in even taxonomically-related face (*Musca autumnalis*) or stable flies (*Stomoxys* spp., Muscidae), nor in other insect or animal phyla including strains with pyrethroid resistance. This implies a species-specific group of P450s with an amino acid sequence at the catalytic site divergent from other known P450s. Moreover, the "Rutgers" multi-insecticide resistant house fly strain overexpresses a selectively-induced (phenobarbital, piperonyl butoxide) non-pyrethroid associated P450 distinct from $P450_{lpr}$, and this *CYP6A1* gene in turn is overexpressed in the LPR strain (see chapter **3**, *46*). These studies clearly illustrate the incredible biochemical diversity of insects, and also suggest limitations to use of a defined molecular probe of resistance for study of a seemingly parallel resistance in even the same species, let alone unrelated animal species.

Biochemodiversity is also evident in P450s adapted to natural insecticides. The *CYP6B1* gene encoding a cytochrome P450 in *Papilio polyxenes* is overexpressed and selectively induced by xanthotoxin feeding (see chapter **9**). Closely related *P. brevicauda*, another Apiaceae specialist, exhibits mRNA that hybridizes with *CYP6B1*,

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but more distantly related *Papilio* species that feed on a wide variety of host plants or on furanocoumarin-deficient diets lack orthologues to this gene. Moreover, a distantly related lepidopteran, the parsnip webworm, *Depressaria pastinacella*, an Apiaceae specialist that consumes up to 7% of its body weight in furanocoumarins per day, has no cross-reactive P450s. Specific P450 genomes limited to species or genera that elicit resistance (tolerance) to compound classes may be the norm instead of the exception among insects.

Separate P450s are implicated in pyrethroid and benzoylphenylurea resistance in diamondback moth, *Plutella xylostella* (see chapter **12**). Moreover, resistance to the P450 inhibitor piperonyl butoxide is also associated with P450 monooxygenases. Interestingly, in this species P450-based resistances do not result in cross-resistance to organophosphates possibly due to enhanced P450 activation of the phosphorothion to the oxon. This may explain why rotation to this latter class of insecticides is successful as a short-term resistance management strategy against multiresistant strains of larval *Plutella* (*47*).

**More Conservatism Among Glutathione Transferases?** Structural changes in glutathione transferase (GST) genes resulting in insecticide resistance have also been delineated. Overexpression of glutathione transferase is implicated in malathion resistance in *Drosophila* (see chapter 5). Elevated transcription of DM GST1 resulting in enzyme with two amino acid changes is the predominant difference between the resistant and susceptible strains. However, pleitropic effects including enhancement of another form of glutathione transferase and a cytochrome P450 indicates a polygenic, regulatory mechanism is also operating.

Clear associations between natural and synthetic insecticide susceptibilities and glutathione transferases have been found among polyphagous Lepidoptera. Larvae feeding on plants containing isothiocyanates possess corresponding isothiocyanate-inducible and -metabolizing glutathione transferases (see chapter **14**). Polyphagous species contained more GST forms and were more individually responsive to the induction of this enzyme than a specialist species, the velvetbean caterpillar, *Anticarsia gemmatalis*. Moreover, allelochemical induction of these GSTs increased the insect's tolerance to organophosphates known to be detoxified by these enzymes (*48*).

In comparison of wild populations of a polyphagous pest, tufted apple bud moth *Platynota idaeusalis*, from managed versus adjacent natural sites, only elevated glutathione transferase consistently correlated with incidence of azinphosmethyl resistance in the adult (see chapter **13**). The use of DM GST1 as a probe of glutathione transferase-based resistance in this species should be successful because of the highly-conserved coding regions of this genome among different organisms.

**Target Site Comparisons**. *Kdr* in house fly (see chapter **6**) and German cockroach, *B. germanica* (see chapter **18**), and cyclodiene resistance in *Drosophila* (see chapter **7**) are probably conferred by structural changes in the receptor rather than decreased channel densities. Similarly, reduced affinity at a *Drosophila* binding protein/receptor for juvenile hormone analogues (see chapter **8**) and *B. thuringiensis* (see chapter **15**) best explains the respective resistance. For the latter, independent receptors in gut brush border membranes for different *Bt* insecticidal crystal proteins have been found in Indian meal moth, *Plodia interpunctella*, and diamondback moth (*36*). Receptor resistance to binding of one toxic protein such as CryIA does not confer cross-resistance to others such as CryIC, thus formulations or transgenic plants with multiple insecticidal proteins may present a useful management tactic to retard resistance development.

Large reductions in target site-sensitivities to insecticides may be best explained by synergistic interaction of dual mechanisms at the site of action. *Kdr* and super-*kdr* resistance to pyrethroids and DDT has been suggested to involve both a site 6 voltage sensitive sodium-channel effect and a presynaptic effect on calcium regulation, respectively (see chapter **6**). Interestingly, *kdr* mediated cross-resistance occurs to some of the allosterically-coupled sodium channel toxins such as veratridine, aconitine and grayanotoxins but not all (e.g. N-alkylamides, batrachotoxin) of these site 2 ligands. In comparison, *kdr* resistance to pyrethroids in German cockroach provides some cross-resistance to batrachotoxin and aconitine, but only a resistance ratio of 2 is found for veratridine (see chapter **18**; *49*). Alternatively, the dual synergistic effects of moderate insensitivity combined with detoxication at the nerve target site may interact to confer cyclodiene resistance in western corn rootworm, *Diabrotica virgifera virgifera* (*50*).

Indirect conformational effects at other binding domains on the sodium channel may allow resistance management via channel-directed chemistry that can still productively bind to what is otherwise an insensitive target. N-Alkylamides are prime candidate insecticides for insects with *kdr*. By acting at a site 2 domain that by exception is not allosterically coupled to the pyrethroid site (see above and chapter **22**), this insecticide class demonstrates no cross-resistance to *kdr* (*51*). Integration of similarly acting insecticides to delay target site resistances will require QSAR of not only the primary binding site but also of the coupled domains and non-interacting sites in both susceptible and resistant strains of the targeted pest.

At present, much of the QSAR to determine the mechanism and optimize the action of insecticides has been performed with mammalian tissues. While this is often necessary because of tissue limitations, better microtechniques need to be developed so that functional nerve ion channel and binding assays can be performed on targeted pests. For example, while stimulation of sodium uptake through mouse brain synaptoneurosome channels corresponded at least qualitatively with insecticidal efficacy of the N-alkylamide, the potency to displace a site 2 radioligand in the same brain preparation was not correlated with insecticidal effect (see chapter **22**). Knowledge of the membrane environment and the three-dimensional amino acid domains in the quaternary structure of the channel assembly that is responsible for functional binding will facilitate QSAR optimization of new generation insecticides.

**Multiple and Other Resistances.** Multiresistance to organophosphates and carbamates in Baygon-R German cockroach is correlated with oxidative and hydrolytic metabolism and reduced penetration (see chapter **18**). However, plural mechanisms available to multiresistant insects may not necessarily provide resistance to new classes of insecticides. Even a field population of Colorado potato beetle multiresistant to most synthetic insecticides remained fully susceptible to the natural insecticide, 2-tridecanone (*52*). Novel insect growth regulators (IGRs) from among the benzoylphenyl ureas and juvenile hormone analogues effectively manage a variety of multiresistant coleopteran and lepidopteran pests; subsequent resistance is associated with hydrolytic and oxidative metabolism and decreased penetration (see chapter **19**, *53*). Among Diptera, exemplified by IGR cross-resistance in house fly (chapter **19**) and methoprene resistance in *Drosophila* (see chapter **8**), target-site insensitivity may be more common than detoxication as a primary mechanism of resistance.

Similar tendencies have been found with abamectin resistance. Cross-resistance in multiresistant and even cyclodiene-resistant (share GABA-action but with non-allosterically interacting binding domains) arthropods to abamectin is low or absent. In Colorado potato beetle, piperonyl butoxide-suppressible oxidative metabolism dominates over carboxylesterase involvement in conferring resistance (see chapter **20**), whereas alterered binding and a 2.4 fold decreased rate of penetration is associated with >60,000 abamectin resistance in house fly (*37*). Nevertheless, cross-resistance to abamectin in the LPR strain of house fly was due to metabolism and decreased penetration (*54*).

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While increased dietary exposure to natural insecticides such as plant defensive chemicals and mycotoxins may be hypothesized to explain the apparent dominance of detoxication over target site resistances in herbivorous Coleoptera and Lepidoptera compared to that in omnivorous and blood-sucking Diptera, some bias may result from differences in methods used to select resistant strains. Most studies with non-herbivorous Diptera use lab strains thereafter selected with topically-applied insecticides, whereas many coleopteran and lepidopteran studies resort to field-resistant populations presumably exposed to insecticides primarily by ingestion. More definitive study is required to determine the role of selection dynamics (e.g. routes of exposure, inbred lab versus field strains) in altering propensity for detoxication versus target site insensitivity in resistance development.

Adaptation to host plant allelochemicals and incidental dietary toxicants such as mycotoxins or phytoalexins are closely related to insecticide resistance development (*55*), and similar mechanisms of gene selection and expression may be operating for both. *Spodoptera frugiperda* is more tolerant than *Helicoverpa zea* to aflatoxin $B_1$ and griseofulvin because of its higher tendency to detoxify than oxidatively activate these mycotoxins, while fungus-feeding sap beetles (*Carpophilus hemipterus*) are more tolerant to trichothecenes than either of these two species due to greatly enhanced hydrolytic detoxication (see chapter **21**). In *H. zea*, different P450 monooxygenases have been associated with resistance to or induction by a specific allelochemical based on substrate specificity studies (see chapter **11**). Understanding these complex natural interactions will be seminal to integration of host plant resistance, including that to diseases, with insecticides in pest management strategies.

Complicating the identity of resistance mechanisms in the *Diabrotica* spp. complex is a behavioral component; avoidance of GABA-acting antifeedant terpenoids by rejecting marginal host plants may allow cyclodiene resistance to be maintained in western corn rootworm. Conversely, feeding by the northern corn rootworm, *D. barberi*, on plants containing picrotoxinin-like epoxides may select for GABA-dependent channel sites with altered binding domains that accommodate cyclodienes no longer used in corn pest management. A link between antifeedant sensitivity at gustatory chemosensory neuroreceptors and resistance to cyclodienes at more internal GABA receptors is suggested (see chapter **23**). Vertebrate probes for $GABA_A$-like cDNA have not been useful for detecting the pharmacologically-divergent (*56*) insect GABA subunits; indeed a number of different receptor subunit variants for this ligand-gated channel may be produced at the same gene locus (see chapter **7**). This species-tissue specificity may limit receptor detection by foreign GABA-channel probes.

## Conclusions

Resistance is the major factor that limits use of chemicals in insect control (*1*, *57*). Although the focus of this book is on studies to identify the molecular basis of resistance, another important aspect of this field will continue to be interactions between resistance mechanisms/genes. Originally, based on isolation of resistant lines, interactions were thought to be largely multiplicative (*58*). However, today these original experiments have other genetic explanations (i. e. *trans*-acting regulatory factors), and multiplicative interactions probably do not hold for similar resistance mechanisms (i. e. two different detoxication mechanisms, *59*).

Resistance mechanisms to insecticides/acaricides (*60*) have been characterized in only a few beneficial arthropods (*61*), and only preliminarily addressed at the molecular level (*62*). Such study is critical for preserving natural enemies in IPM programs where pesticides are used. Insertion of resistance-conferring genes via baculovirus recombinants (*63*) into key beneficials is one approach to this problem. The agrochemical industry must continue screening insecticides in development on

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multiresistant strains of insects and representative entomophagous insects to identify problematic chemistry.

While it is easy to get caught up in the marvel of resistance - the fact that a given strain can survive several thousand fold more toxicant than a "normal" strain - and the amazing biochemical and genetic traits that allow for this survival, it is important that we keep in mind that resistance must address practical aspects as well. In this book we have tried to present an overview of all these different areas. We feel that it is important for the transfer of information back and forth between investigators working on model systems such as *Drosophila* and those working on agriculturally and medically important insects and Acari. The rapid pace of discovery of molecular mechanisms of resistance among different species will only be assured if critical sharing of gene probes among labs is maintained. Only through this type of communication can the field as a whole go forward.

Prolonging the lifetime of a pesticide, whether introduced transgenically or exogenously for insect control, should be a primary goal of both agrochemical industry and production agriculture. Mixtures of active compounds and their synergists can be quite useful in delaying resistance, and insecticides with multiple, selective sites of action may dominate control strategies of the future. Pivotal for chemical implementation will be our understanding the dynamics of the molecular basis of insecticide resistance.

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# Chapter 2

# Characterization of a Cytochrome P450 Responsible for Pyrethroid Resistance in the Housefly

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We have recently purified a cytochrome P450, termed P450$_{lpr}$, from the insecticide resistant LPR strain of house fly (*Musca domestica*). A polyclonal antiserum was raised, shown to be specific for P450$_{lpr}$ and used to characterize this P450. P450$_{lpr}$ was expressed at highest levels in adult LPR house flies, compared to larvae or pupae, and was found in both sexes. P450$_{lpr}$ was found at 44-fold higher levels in LPR than in susceptible flies. A single immuno-reactive band in denaturing gel electrophoresis, corresponding to P450$_{lpr}$, was found in all house fly strains tested, with higher levels being found in resistant compared to susceptible strains. Anti-P450$_{lpr}$ inhibited P450 monooxygenase dependent *in vitro* deltamethrin metabolism in LPR microsomes indicating that P450$_{lpr}$ is involved in P450 monooxygenase dependent pyrethroid resistance.

## Cytochrome P450 Monooxygenases

The microsomal cytochrome P450 dependent monooxygenases (hereafter called "P450 monooxygenases") are an extremely important metabolic system. P450 monooxygenases are important in the detoxication of xenobiotics such as drugs, pesticides, and plant toxins; and in the regulation of endogenous compounds such as hormones, fatty acids, and steroids. P450 monooxygenases are found in almost all aerobic organisms, including organisms as diverse as plants, insects, mammals, birds, and fungi. In eukaryotes, P450 monooxygenases are typically found in the endoplasmic reticulum of metabolically active tissues. The two most important components of the P450 monooxygenase system are cytochrome P450, which acts as the substrate binding protein (and terminal oxidase), and NADPH-cytochrome P450 reductase (P450 reductase), which transfers electrons from NADPH to cytochrome P450. Cytochrome b$_5$ may have a role in P450 monooxygenase activity by donating the second electron or by modulating P450 monooxygenase

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activity through inhibitory or stimulatory interactions with P450 *in vitro* (1,2). However, the exact role of cytochrome b5 is still questioned because it is not required for activity in most reconstituted P450 monooxygenase systems. The role of cytochrome b5 in P450 monooxygenase activity in insects is poorly understood.

P450 monooxygenases are capable of oxidizing a bewildering array of xenobiotics (3). This remarkable breadth of utilizable substrates is due to the large number of cytochrome P450 forms that are expressed in each organism. For instance, over 13 P450s have been isolated from rats (4) and 154 cytochrome P450 cDNA sequences have been described mostly from mammalian systems (5). The specificity of the P450 monooxygenase system, therefore, is dependent on the P450 cytochrome(s) present, many with apparently overlapping specificity. This complexity requires that individual cytochrome P450 forms be isolated in order to understand and characterize their contribution to important metabolic functions.

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**Role of P450 monooxygenases in insects.** The P450 monooxygenases of insects have several functional roles, including growth, development, feeding, resistance to pesticides, and tolerance to plant toxins (6). Furthermore, P450 monooxygenases are intimately involved in the synthesis and degradation of insect hormones and pheromones including 20-hydroxyecdysone and juvenile hormone (6).

Insect P450 monooxygenases can be detected in a wide range of tissues. Highest P450 monooxygenase activities are usually associated with the midgut, fat bodies, and Malpighian tubules (7). Dramatic variation in the levels of cytochrome P450 and monooxygenase activity are seen during the development of most insects (7-9). In general, P450 levels are undetectable in eggs, rise and fall in each larval instar, are undetectable in pupae, and are expressed at high levels in adults (6).

**Role of P450 monooxygenases in pesticide resistance.** The role of monooxygenases in insecticide resistance first became apparent in the early 1960s, when Eldefrawi et al. (10) showed that resistance to carbaryl could be abolished by the methylenedioxyphenyl cytochrome P450 inhibitor sesamex. Additional evidence of monooxygenase based resistance quickly accumulated (11-13). We now know that insects commonly become resistant to insecticides due to increased detoxication mediated by the cytochrome P450 monooxygenase system. This resistance mechanism is very important because it can confer both high levels of resistance (14,15) and may also confer cross-resistance to unrelated compounds due to the breadth of substrates the P450 monooxygenases can metabolize (16). Furthermore, P450 monooxygenase-mediated detoxication has been found as a mechanism of resistance in a large number of important pests (17).

How genetic selection for increased monooxygenase metabolism of insecticides comes about is a major question in resistance research; work that has been carried out almost exclusively in house flies. Major questions include how "resistance" monooxygenases are expressed, and what is different about these monooxygenases that causes increased metabolism of insecticides. Research historically has taken four approaches to answer these questions: 1) enzymology studies to determine the metabolic differences between resistant and susceptible strains of house flies, 2) physical studies, including spectral studies, to determine the nature of P450s in resistant and susceptible house flies, 3) linkage studies to examine the genetic control of monooxygenase-mediated resistance in different resistant strains of house flies, and 4) molecular genetic approaches aimed at cloning of resistance related P450 genes.

**Metabolic studies on cytochrome P450 monooxygenase dependent resistance in house flies.** In 1967 Tsukamoto and Casida (18) showed that carbamate resistant house flies exhibited increased ability, compared to susceptible flies, to perform oxidative hydroxylations, *N*-demethylations, *O*-

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demethylations, epoxidations and desulfurations. Soon after, other workers showed the diazinon resistant Fc strain had increased ability to oxidize DDT (19), aldrin and naphthalene (20). Increased ability to metabolize many substrates was shown in other insecticide resistant house fly strains such as Rutgers (21). In addition, increased levels of total cytochrome P450 were found in several insecticide resistant strains such as Diazinon-R, Fc, R-Baygon, dimethoate-R, Orlando-R, Malathion-R and Ronnel-R (refs. in 3).

Although increased cytochrome P450 monooxygenase activity can be correlated with increases in resistance (6), lack of correlation between increased total cytochrome P450 and *in vivo* monooxygenase has been noted in some cases (3, 22, 23). Increases of 2- to 3-fold in cytochrome P450 have been noted, while monooxygenase activity can increase as much as 64-fold (24). *In vitro*, monooxygenase based resistance does not always correlate with increases in all types of monooxygenase activities (17, 23). Schonbrod et al. (20) showed no correlation between *in vitro* oxidase activity and resistance patterns in 14 strains of house flies using two oxidase assays.

The eventual realization that there were multiple forms of cytochrome P450 in insects, coupled with the realization from mammalian research that these might be regulated independently from each other, led to the modern interpretation of these data. In an analogous situation, it is known that phenobarbitol induction in rats can increase the levels of one cytochrome P450 24-fold, thereby increasing the total cytochrome P450 level 3-fold (25). Similarly, some types of resistance are thought to be due to an increase in a subfraction of the total cytochrome P450 pool (6). Wilkinson (17) has pointed out that choice of assay is a general problem with metabolic research into monooxygenase resistance. There is the danger that poor correlation of multiple resistance patterns and metabolic assay could result from using a particular assay for monitoring monooxygenase activity while a "resistance" cytochrome P450 is not the major user of that substrate.

Early studies showing lack of correlation between cytochrome P450 levels and resistance led some workers to conclude that cytochrome P450 may not be the limiting factor in monooxygenase dependent resistance (22). These studies have been reinterpreted to account for multiple P450s now known to exist in insects (3). In theory, monooxygenase dependent resistance can be due to increased levels of a minor P450 that does not significantly increase the levels of total cytochrome P450 (23). Cases to support this theory have not been demonstrated due to lack of knowledge about individual cytochrome P450s in insects.

**Physical differences in cytochrome P450 from resistant and susceptible house flies.** Hodgson and Kulkarni (26) have shown that microsomes from resistant flies contain "different" cytochrome P450 compared to susceptible flies. Using off-balance spectral studies they showed two classes of cytochrome P450, resistant strains having an absorbance peak at 394 nm while susceptible flies had a cytochrome P450 absorbance peak at 412 nm. Experiments using controlled tryptic digestion of microsomes or density gradient centrifugation showed multiple forms in different strains with different mixtures in different strains. Also the cytochrome P450 from resistant house flies was more accessible to proteolytic digestion than cytochrome P450 from susceptible house flies.

Cytochrome P450 from resistant house fly strains was shown to have one or more of the following characteristics based on the work of Hodgson and others (3). Microsomes from resistant flies tended to show a carboxy ferrocytochrome absorbance maximum several nanometers below that of susceptible house flies, with increased levels of total cytochrome P450 assayed by this method. Significant type I binding was present (a measure of the capacity to bind lipophilic substrate) in resistant house fly microsomes. In addition, in some strains there were subtle alterations in type II and III spectra (measures of the capacity to bind compounds that substitute for the sixth Fe axial ligand (histidine) and oxygen, respectively). Interpretation of these changes is difficult. Clearly, resistant house flies have P450(s) different from or in addition to the complement from susceptible flies, how these differences relate to the expression of resistance is not understood.

**Linkage studies on resistant house flies.** Resistant house fly strains with increased levels of monooxygenase activities, broad cross-resistance and increased total cytochrome P450 levels are known. Two or more semi-dominant genes that express high oxidase levels are known for house flies on chromosomes II and V (refs. in 3), with high oxidase activity segregating with autosome II being more common. In 1986, Scott and Georghiou (27) showed monooxygenase mediated resistance associated with autosome I in the pyrethroid resistant LPR strain. Plapp (28) has suggested resistance is often due to an altered regulatory region on autosome II that could regulate several xenobiotic metabolizing enzymes simultaneously. However, Wilkinson (17) argued that since metabolic cross resistance varies widely among resistant strains, resistance must be due to different P450s under separate control. It is not presently known whether either of these hypothesis are correct, however there is at least one case where high levels of monooxygenase-mediated resistance are not associated with chromosome 2 (27).

**Cloning of resistance related P450 genes.** This type of approach to the study of the P450 monooxygenases is relatively new and holds great promse for improving our understanding of the regulation of P450 genes. The chapters in this book by Carino et al. and Waters et al. provide excellent information on the important advances being made in this area.

**Purification of cytochrome P450 from insects.** The foregoing discussion suggests that three major questions can be addressed concerning insect cytochrome P450 monooxygenases: 1) how are insect P450s different or similar to the better studied vertebrate P450s, 2) how are resistance related cytochrome P450s different from susceptible cytochrome P450s and 3) what is the genetic basis for monooxygenase dependent resistance? It has long been realized that purification of cytochrome P450 forms from insecticide resistant insects and development of antisera to them would be needed to investigate these important questions about the biochemical and genetic basis of insecticide resistance (3, 6). This point was not lost on earlier workers and several attempts have been made to isolate an insect P450, with resistant house flies being the preferred starting material (29-32).

Purification of a P450 from insects in useful quantity and quality remained elusive. The difficulties encountered in insect cytochrome P450 purification have been detailed (3, 31). Schonbrod and Terriere (33) reported resolution of two forms of low specific content (impure or damaged cytochrome P450 preparations), but produced early evidence of multiplicity of cytochrome P450 in house flies. An early attempt (34) using uninduced, susceptible house flies was remarkably successful, isolating one P450 with high specific content (13.9 nmol cytochrome P450/mg protein), but impure based on gel electrophoresis. This method required seven open-column chromatographic steps and apparently was not pursued further. The resolution of several more low specific content impure cytochrome P450 preparations were reported by Yu and Terriere (29). Moldenke et al. (30) resolved two crude cytochrome P450 fractions and could reconstitute the oxidase activity with purified cytochrome P450 reductase to show that different cytochrome P450

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fractions have different metabolic capabilities. In the same year Fisher and Mayer (31) reported a partially pure preparation from the Rutgers diazinon resistant strain with moderate specific content (10 nmol/mg) and partially characterized it. Recently Ronis et al. (32) partially purified several P450s from the Rutgers strain with specific contents ranging between 2.5-7 nmol/mg and showed that they could, with limited success, be reconstituted with mammalian cytochrome P450 reductase. In addition, phosphotidylethanolamine was better in their reconstitutions than phosphotidylcholine. This result is consistent with phosphotidylethanolamine being the major phospholipid in insects. Recently, Sundseth et al. (35) reported purification of a constitutive P450 with low specific content from Drosophila and partial purification of a resistance-correlated P450 from insecticide resistant Drosophila. However, a biochemically useful purification of a cytochrome P450 from an insect and more importantly, from an insecticide resistant house fly, remained elusive, despite the great advances that would be obtained from such a probe.

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## Scope of the Chapter

To effectively study the biochemistry of P450 monooxygenase-mediated resistance it is necessary to isolate the P450(s) involved. Historically, two major problems were encountered when attempts were made to characterize insect cytochrome P450s: the limited amount of starting material available, and the apparent lability of microsomal preparations from insects. This chapter will describe our efforts over the last few years to characterize a cytochrome P450 from house flies that is involved in resistance to pyrethroid insecticides.

## History of the LPR Strain

The Learn Pyrethroid Resistant (LPR) strain of house fly was originally collected in 1982 from a dairy in New York and had low levels of resistance to a broad range of insecticides. After laboratory selection with permethrin for 22 generations (36), the LPR strain became homozygous for the major mechanisms of resistance and attained extremely high levels of resistance to pyrethroid insecticides with a phenoxybenzyl alcohol moiety (ex. 6,000-fold permethrin resistance and >100,000-fold deltamethrin resistance, (15)).

There is considerable evidence for the role of P450 monooxygenases in pyrethroid resistance in the LPR strain. First, permethrin resistance can be reduced from 6000- to 33-fold by the P450 inhibitor piperonyl butoxide (15). Second, *in vitro* studies reveal that P450 monooxygenase-mediated detoxication of permethrin occurs at a rate nearly 10-fold greater in LPR compared to the susceptible strain with a preference for the *cis* isomer compared to the *trans* isomer (15). Third, compared to wild-type flies LPR has increased P450 monooxygenase-mediated methoxyresorufin *O*-demethylase (64-fold), ethoxyresorufin *O*-deethylase (56-fold), ethoxycoumarin *O*-deethylation (11-fold), and aldrin epoxidation (8.4-fold) activities (24).

Adult LPR flies also contain abnormally high, constitutively expressed levels of the P450 monooxygenase components compared to susceptible wild-type flies. Cytochrome P450, P450 reductase, and cytochrome $b_5$ are 4-, 3-, and 2-fold higher in LPR, respectively (15). In fact, these levels are higher than found in other resistant strains (23) and compare favorably with mammalian P450 sources. Additionally, the absorbance maximum is shifted from 452 nm in wild-type to 450 nm in LPR flies, suggesting the levels of individual P450s may be different between these strains.

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SDS-PAGE analysis of house fly microsomes revealed the presence of a 54.3 kDa protein that was phenobarbital inducible in the susceptible strain (characteristic of total cytochromes P450 in insects) and expressed at a substantially higher level in LPR compared with susceptible flies (24). This protein has been the subject of further studies described below.

It has recently been discovered that lack of control with pyrethroid insecticides in *Heliothis armigera*, a severe cotton pest in Australia, is due to elevated P450 monooxygenase-mediated detoxication (37). Interestingly, the P450 monooxygenase-mediated cross-resistance was limited primarily to phenoxybenzyl pyrethroids. These results are nearly identical to the cross-resistance patterns found in the LPR house flies (15). In fact, the results from the house fly studies have provided the basis for much of the work on *H. armigera* (37), especially in the search for pyrethroids that might not be affected by the P450 monooxygenase-mediated resistance mechanism.

The foregoing data suggest LPR is a good model to study insect P450 monooxygenases in general, and P450 monooxygenase-mediated insecticide resistance specifically. The qualitative differences in cytochrome P450, and the constitutive and abundant expression of cytochrome P450 argues that the LPR strain is resistant to pyrethroids due to a single or limited number of P450s that are abnormally regulated in LPR. The development of a buffer system that stabilized the cytochrome P450 monooxygenases (38) allowed us to pursue the characterization of individual cytochrome P450s.

## Purification of Cytochrome $P450_{lpr}$

In 1989 we reported the purification of a major cytochrome P450, termed $P450_{lpr}$, from LPR house flies to apparent electrophoretic homogeneity using two high performance liquid chromatography (HPLC) steps (39). This P450 runs as a single band at 54.3 kDa by SDS-PAGE, corresponding to a major band in LPR, and a phenobarbital inducible band in wild-type (susceptible) flies. It has a carboxy ferrocytochrome absorbance maximum at 447 nm with no apparent peak at 420 (i.e., no denatured P450), has a high specific content (14.4 nmol/mg protein), and can be readily isolated in substantial quantities (39). The N-terminal amino acid sequence of $P450_{lpr}$ is Met-Leu-Leu-Leu-Leu-Leu-Leu-Ile-Val-Val-Thr-Thr-Leu-Tyr-Ile (Wheelock and Scott unpublished). This sequence shares no homology with published P450 sequences. We believe $P450_{lpr}$ is a single cytochrome P450, since it cannot be resolved into multiple components chromatographically, immunologically or electrophoretically (40).

## Production and Characterization of $P450_{lpr}$ Antisera

A polyclonal antiserum (8-241) was raised in rabbits using purified cytochrome $P450_{lpr}$ protein as the antigen. Several dilutions of both purified $P450_{lpr}$ and LPR microsomes produced single sharp immunoprecipitin bands in rocket immunoelectrophoresis (RIE) (40) using antiserum 8-241. Thus, the antiserum reacted with a single antigen in samples of both LPR microsomes and purified cytochrome $P450_{lpr}$. Evidence the antigen in both purified $P450_{lpr}$ and LPR microsomes was identical was provided by fused RIE, where precipitin lines from both purified $P450_{lpr}$ and LPR microsomes fused without any discernable spurs, showing a reaction of identity (40).

Cytochrome P450s exist as a mixture of related forms with conserved regions, but different electrophoretic and/or chromatographic properties (4). Since the above data could not exclude the possibility that the antiserum consisted of

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antibodies against conserved regions possessed by multiple LPR cytochrome P450s, we performed two additional immunological tests: SDS PAGE/immunoblotting and ion exchange chromatography/RIE. In these experiments cytochrome P450s were separated prior to immunological challenge in order to detect any immunologically identical, but chromatographically different cytochrome P450s.

SDS-PAGE followed by immuno-blotting with antiserum 8-241 revealed LPR microsomes had a single immunoreactive band at the position of P450$_{lpr}$ (40) suggesting that P450$_{lpr}$ was immunologically distinct from any other microsomal proteins. DEAE ion exchange HPLC, which resolves other partially purified cytochrome P450s from LPR (39), followed by RIE of the HPLC fractions showed that only the fraction containing P450$_{lpr}$, contained a strongly immunoreactive protein (40). We conclude from these studies that antisera 8-241 reacts with an immunologically distinct antigen in crude LPR house fly microsomes that is immunologically identical to purified P450$_{lpr}$, and that the antisera is specific for cytochrome P450$_{lpr}$ under the conditions used.

## Expression of P450$_{lpr}$

**Quantitation of P450$_{lpr}$ in LPR and S+ house flies.** The average amount of P450$_{lpr}$ in LPR microsomes, as a percentage of the total P450, was determined to be 68% by quantitative RIE (40), suggesting that P450$_{lpr}$ was the major cytochrome P450 in LPR microsomes. Microsomes were also immuno-assayed in the insecticide susceptible S+ strain. Immunoreactive cytochrome P450 was found to be a minority of the total cytochrome P450 in S+, comprising only 6.5% of the total. Also, the total amount of cytochrome P450 in LPR is much higher than in S+, consistent with previous results (15, 24). A calculation from the specific contents results in an estimation of 44-fold higher levels of immunologically reactive cytochrome P450 in LPR microsomes compared to S+ microsomes (40).

An intriguing question is whether resistance associated cytochrome P450s represent mutant forms of cytochrome P450 (i.e. a form not normally expressed in susceptible strains) or increased levels of a particular form(s) normally present in susceptible strains. Sundseth et al. (41) showed in *Drosophila melanogaster* that a resistance associated cytochrome P450 was detectable, but at levels 10-20 times lower in susceptible compared to resistant strains, although they did not quantitatively assay cytochrome P450 levels in either strain. Our finding of a cytochrome P450 that is immunologically, electrophoretically and chromatographically identical to P450$_{lpr}$ expressed at 44-fold higher levels in resistant LPR house flies compared to susceptible house flies (40) supports the hypothesis that resistance related P450s can be increased levels of constituitively expressed P450s.

**Presence of P450$_{lpr}$ in other house fly strains.** Microsomes from insecticide resistant and susceptible house fly strains were evaluated for the presence of P450$_{lpr}$ using SDS-PAGE/immuno-blotting. Single immunoreactive bands were found in the resistant strains (LPR, Dairy, Kashiwagura, 3rd-Y, EPR, ASPR$_m$, ASPR$_f$ ) while the susceptible strains (*aabys*, S+) showed only a weak reaction (40). Additionally, the immunoreactive bands had the same mobility in LPR, *aabys*, S+, Dairy, 3rd-Y, EPR and ASPR$_f$ compared to the standard P450$_{lpr}$, while Kashiwagura and ASPR$_m$ bands had slightly increased mobility. Although it is clear from this result that these house fly strains contain an antigen that shares at least one antigenic determinant with P450$_{lpr}$, the immuno-blotting technique could not differentiate between an antigen identical to P450$_{lpr}$ and antigens with only partial homology. Therefore, we used fused RIE with purified

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$P450_{lpr}$ as the reference antigen to assess the immunological identity of these antigens present in the various house fly strains. Cytochrome P450s were isolated and fractionated from LPR, *aabys*, S+, Dairy, Kashiwagura, 3rd-Y, EPR, $ASPR_m$ and $ASPR_f$ using hydrophobic interaction and ion exchange HPLC. The fractionated P450s from the ion exchange step were assayed for immunoreactivity by RIE. The results for each strain revealed a major cytochrome P450 peak associated with immunoreactivity. The position of the major immunoreactive peak was approximately 13 ml post injection in LPR, Dairy, 3rd-Y, EPR, *aabys* and S+, but approximately 12 ml post injection in Kashiwagura, $ASPR_m$ and $ASPR_f$ (40).

The immunoreactive fractions from the HPLC experiment showing immunoreactivity were compared for immunological relatedness to $P450_{lpr}$ using fused RIE. All tested fractions fused completely with $P450_{lpr}$, indicating immunological identity with $P450_{lpr}$ (40). These results are in agreement with those of Sundseth et al. (41) who used monoclonal antibodies to show that three resistant Drosophila strains contained cytochrome P450(s) that contained a common single immunogenic epitope. In the present study, fused RIE using a multivalent polyclonal antiserum revealed that antigens from other resistant house flies were immunologically indistinguishable from cytochrome $P450_{lpr}$. This is a more rigorous test of homology than the presence of a single shared epitope since fused precipitin formation as in RIE requires multiple shared epitopes (42). Our fused RIE results, combined with the slightly different electrophoretic migration of immunologically reactive P450 in the Kashiwagura and $ASPR_m$ microsomes and the different chromatographic retention times of Kashiwagura, $ASPR_m$ and $ASPR_f$ compared to $P450_{lpr}$ suggest that they are closely related to $P450_{lpr}$ but may contain subtle differences, while antigens from *aabys*, S+, Dairy, 3rd-Y and EPR were indistinguishable from $P450_{lpr}$ electrophoretically, chromatographically, and immunologically (40).

It is remarkable that all resistant strains tested had elevated levels of a single immunoreactive antigen per mg microsomal protein compared to susceptible strains. Two of the resistant strains used (3rd-Y, EPR) were susceptible to pyrethroids but resistant to organophosphates, three were moderately, (Kashiwagura, ASPR) or highly (LPR) resistant to pyrethroids, and one was multiresistant (Dairy). Thus, there was no discernable correlation with presence of immunoreactive antigen and resistance to a class of insecticide. This suggests that the P450 that is immunologically, electrophoretically and chromatographically identical to $P450_{lpr}$ in 3rd-Y, EPR and perhaps some of the other strains is catalytically different from $P450_{lpr}$ (i.e. $P450_{lpr}$ metabolizes deltamethrin (see below) while these other very similar P450s may not). Unfortunately, the functional characteristics that distinguish resistance related cytochrome P450s remain poorly understood (3).

How can closely related cytochrome P450s have different substrate specificities? The closely related rat cytochrome P450b (P450IIB1) and P450d (P450IIB2) have been shown to differ by only 13 out of 491 amino acid residues (97% homology), but can be chromatographically resolved (4) and have markedly different catalytic properties. Recent studies of the effect of engineered point mutations on P450cam (43, 44) demonstrate that single amino acid changes around the catalytic site of cytochrome P450s can radically alter substrate regiospecificity. Similarly, different house fly strains could have resistance related cytochrome P450s with minor sequence differences that would result in different catalytic capabilities, but would not be differentiated by immunological tests.

**Cytochrome $P450_{lpr}$ expression in LPR house flies.** Microsomes from 3-5 days old adult male or female LPR house flies were found to contain immunostaining P450 at the position of authentic $P450_{lpr}$, showing that cytochrome

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$P450_{lpr}$ is expressed in both male and female adult LPR house flies (45). Microsomes from males had a specific content of 0.53 nmol $P450_{lpr}$/total cytochrome P450 or 0.37 nmol $P450_{lpr}$/mg protein. Microsomes from female flies of the same three rearings had 0.53 nmol $P450_{lpr}$/nmol P450 or 0.14 nmol $P450_{lpr}$/mg protein. Thus, $P450_{lpr}$ represented the same fraction of total P450 in both female and male microsomes, but less on a per mg protein basis in females (45).

It appears that cytochrome $P450_{lpr}$ is developmentally regulated in the LPR strain (45). Microsomes were prepared from flies of various ages and then probed with anti-$P450_{lpr}$ in immuno-blots. $P450_{lpr}$ was present in adults of all ages, from 0-3 hr to 5-6 days post emergence as detected by SDS-PAGE immuno-blotting. In contrast, microsomes from 1, 2, 3, 4, 5, and 6 days old LPR larvae revealed no immuno-staining material corresponding to $P450_{lpr}$ in any age (45). $P450_{lpr}$ began to be expressed at barely detectable levels early in pupal development (i.e., between 24-48 hrs. after pupation), and was present at low levels in all pupal stages thereafter (45). Therefore, it appears that $P450_{lpr}$ is first synthesized in pupae with significant $P450_{lpr}$ expression limited to adults, but not otherwise sex or age specific.

Previous reports examining the specific content of total cytochrome P450 (or cytochrome P450 dependent enzymatic activity) have shown only minor differences between adult male and female house flies. Additionally, P450 levels of house fly larvae and pupae are known to be low in comparison with adult levels (6). Our results suggest expression of $P450_{lpr}$ is similar to total P450 in these respects.

Preliminary results indicate that $P450_{lpr}$ is found in many tissies throughout the house fly abdomen (Lee and Scott unpublished). A single immunoreactive P450, with the same mobility as $P450_{lpr}$, has been detected from fat body, reproductive system, proximal intestine, distal intestine and Malpighian tubules of 3-5 day old female LPR house flies. This is an important result because it suggests there is not a single tissue within the house fly that is responsible for resistance.

**Expression of $P450_{lpr}$ in insects, mites, and rodents.** We have examined other animals for the presence of $P450_{lpr}$. Adult face flies, stable flies and fruit flies all gave negative immuno-staining responses, as did larval fruit flies. Representatives of Hymenoptera (honey bee, carpenter ant), Lepidoptera (cabbage looper, tobacco hornworm), Orthoptera (German cockroach) and Acari (two spotted spider mite) did not immuno-stain. PB induction of P450 was obtained in face fly (5.6-fold), stable fly (3.3-fold), fruit fly adults (Canton-S, 1.2-fold; Hikone-R, 1.5-fold), tobacco hornworm (1.3-fold), German cockroach (CSMA, 1.8-fold) and honey bee. This induction did not produce immuno-stainable P450. P450 monooxygenase-mediated insecticide resistant arthropod strains tested include Hikone-R fruit flies, Dursban-R German cockroaches and two spotted spider mites. Insecticide resistance did not confer expression of immunologically recognized cytochrome P450 in these arthropods (45).

Microsomes from rat or mouse liver were tested with anti-$P450_{lpr}$ for cross-reactivity. We could find no immuno-staining band in blots from corn oil treated, 3-methylcholanthrene treated or phenobarbital treated rat liver microsomes. Additionally, no reaction was seen with corn oil treated or benzo(e)pyrene treated mouse liver microsomes (45). We conclude that $P450_{lpr}$ is likely restricted to house flies due to the total lack of cross-reactivity to anti-$P450_{lpr}$ in any of the wide range of species tested.

Among mammals, interspecies homology between P450s as judged by immunological cross reactivities are known (46-48), but the relatedness of insect

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P450s to other species is poorly understood. Based on published insect P450 cDNA sequences, insect P450s have the potential to be unrelated (49, Waters et al. this book) or related (50), both in sequence and function, to P450s from other taxa. Immunological studies of insect P450s have shown little homology with P450s from other classes. Clarke et al. (51) tested house fly microsomes induced with several compounds for reactivity with anti-rat P450 polyclonal antibodies. Although these compounds induced similar cytochrome P450 dependent enzymatic activities in both rats and house flies, these workers could not detect any immunological cross-reaction to house fly microsomes by immuno-blotting. Sundseth et al. (41) examined interspecies cross-reactivity using anti-Drosophila P450 monoclonal antibodies. Using ELISA analysis, they showed that anti-P450-B (against resistance-related P450) cross-reacted with microsomal protein(s) from rat and southern armyworm, but only at the highest concentration used. In contrast, anti-P450-A (against a constitutive P450) only recognized Drosophila microsomes. Cohen et al. (52) probed swallowtail caterpillar microsomes with antisera against non-insect P450s using immuno-blots. They found positive responses to several different bands using antisera against P450s from rat, rabbit, cow, fruit fly and *Pseudomonas putida*.

## Role of $P450_{lpr}$ in Pyrethroid Resistance

**Approach.** When reconstituted with phospholipid and cytochrome P450 reductase, the rate of cytochrome P450 dependent oxidations are greatly influenced by the phospholipid environment and the ratio of reductase to cytochrome P450 (6, 46), and can be stimulated or inhibited by cytochrome $b_5$ (47). In addition, analysis of the reaction products of an isolated cytochrome P450 cannot determine the P450's contribution to a metabolic process in competition with other cytochrome P450 forms. Immunoinhibition affords the only mechanism by which specific P450s can be inhibited in their normal melieu, with the normal phospholipids present in the normal ratios, normal levels of cytochrome P450 reductase and cytochrome $b_5$, competing cytochromes P450 and other metabolic processes that are present. These qualities make this our technique of choice to study the role of individual cytochrome P450s in insecticide metabolism.

***In vitro* deltamethrin metabolism.** To ascertain the role of $P450_{lpr}$ in pyrethroid resistance in the LPR strain we investigated the cytochrome P450 monooxygenase dependent metabolism of deltamethrin. Deltamethrin was chosen because it is a single stereoisomer, all metabolites are less toxic than the parent compound and LPR is highly resistant to it. Microsomes from susceptible or LPR house flies were treated with normal rabbit serum (i.e. the control) or anti-$P450_{lpr}$ antiserum. The results are shown in Table I. These results show that increased levels of cytochrome P450 monooxygenase dependent deltamethrin metabolism occur in LPR house fly microsomes compared to S+ (53). Under these conditions virtually all of the deltamethrin was recovered from the susceptible strain unchanged in both the normal serum and antiserum treatments. However, 24% of the deltamethrin was metabolized by microsomes from LPR flies. This metabolism of deltamethrin could be almost completely inhibited by the anti-$P450_{lpr}$ antiserum. This indicates that $P450_{lpr}$ is the major P450 responsible for deltamethrin metabolism in LPR flies. Whether the remaining 3% deltamethrin metabolism is due to another P450 that is involved in the resistance or simply due to metabolic switching when $P450_{lpr}$ is inhibited is not known.

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Table I. *In vitro* Metabolism of Deltamethrin by Microsomes from Susceptible (S+) and Resistant (LPR) House Flies

| Percent of recovered radiolabel remaining as deltamethrin[a] | | | |
|---|---|---|---|
| S+ | | LPR | |
| NS | AS | NS | AS |
| 100 | 100 | 76 | 97 |

[a] Values corrected for non-specific losses observed in carbon monooxide treated (control) microsomes.
NS = Normal serum.
AS = Anti-P450$_{lpr}$ antiserum.
Data from Wheelock and Scott (53).

Seven resolvable deltamethrin metabolites were detected by TLC. Mobilities of the metabolites relative to deltamethrin (Rd) were statistically different ($p < 0.05$, two-tailed t-test) from each other and identical between strains. All seven metabolites were found *in vitro* with LPR microsomes treated with normal rabbit serum, but were not all found in LPR antiserum treated or S+ microsomes. Treatment of LPR microsomes with anti-P450$_{lpr}$ eliminated metabolite B, and substantially reduced metabolites C and H and the aqueous metabolites (Table II) while metabolite D increased (53).

Table II. *In vitro* Metabolism of Deltamethrin by Microsomes from Resistant LPR House Flies

| | Percent of recovered radiolabel | |
|---|---|---|
| Metabolite | Normal Serum | Antiserum |
| B | 1.7 | 0 |
| C | 3.6 | 1.6* |
| D | 0.9 | 3.1* |
| E | 0.3 | 0.6 |
| F | 0.2 | 0.1 |
| G | 0.4 | 0.3 |
| H | 2.5 | 0.6* |
| Aqueous | 18.7 | 11.0* |

*Significantly different ($P<0.05$).
Data calculated from Wheelock and Scott (53).

**Metabolite identification.** We attempted to identify metabolites by co-chromatography with two available authentic standards. No metabolite co-chromatographed with 4'OH-deltamethrin (there was partial overlap between C and 4'OH-deltamethrin) or 2'OH-deltamethrin (there was partial overlap between this standard and metabolite B) (53).

### Further Substrate Specificity of $P450_{lpr}$

The substrate specificity of $P450_{lpr}$ was evaluated using anti-$P450_{lpr}$ antiserum in four model P450 monooxygenase reactions. Anti-$P450_{lpr}$ inhibited 98% of the methoxyresorufin-*O*-demethylase activity and all the benzo(a)pyrene hydroxylase activity in LPR microsomes, but none of the pentoxyresorufin-*O*-dealkylase activity. The antiserum partially inhibitied ethoxyresorufin-*O*-dealkylase and ethoxycoumarin-*O*-dealkylase activity (53). Based on these data, $P450_{lpr}$ is the major utilizer of benzo(a)pyrene, methoxyresorufin and ethoxyresorufin, but not ethoxycoumarin or pentoxyresorufin in LPR microsomes.

All P450 monooxygenase activities tested are expressed at higher levels in LPR house flies compared to S+ flies: MROD (64-fold), AHH (8-fold), EROD (54-fold) and ECOD (11-fold) (24). MROD and AHH activity were highest in LPR flies compared to other resistant flies and were associated with resistance to pyrethroid insecticides (23). Other insecticide resistant house fly strains contain a cytochrome P450 that is chromatographically, electrophorectically and immunologically indistinguishable from $P450_{lpr}$ at levels intermediate between susceptible and LPR flies (40). These flies also exhibit MROD and AHH activities intermediate between susceptible and LPR flies (23). The present study shows that an antiserum specific for $P450_{lpr}$ inhibits almost all MROD and AHH activity in LPR microsomes. Thus, it appears that MROD and AHH activities are diagnostic for $P450_{lpr}$ in LPR house flies. Whether these P450s can be correlated with MROD and AHH activity in other insecticide resistant strains is an interesting question for future study.

### Immunoinhibition

Immunoinhibition of P450 monooxygenases has been widely studied in vertebrate systems (55-57). However, inhibition can result from inhibition of cytochrome P450 reductase binding or by inhibition of substrate binding at the catalytic site, and can be different for different substrates (58). Non-specific inhibition of P450s via inhibition of the common service protein, NADPH cytochrome P450 reductase, results in inhibition of all P450 monooxygenase reactions (59). This is not so for anti-$P450_{lpr}$ because some reactions are not inhibited (54) and in the case of deltamethrin metabolism, the formation of some P450 monooxygenase produced metabolites are not inhibited or are increased (54). In addition, anti-$P450_{lpr}$ does not inhibit the reduction of cytochrome *c* by P450 reductase (54).

**Mechanism of $P450_{lpr}$ immunoinhibition.** $P450_{lpr}$ was tested for its ability to inhibit cytochrome P450 reductase interaction with cytochrome P450. Reduction of cytochrome P450 was inhibited, up to a maximum of 49%. This inhibition was not due to loss of cytochrome P450 since the remaining cytochrome P450 could be reduced with sodium dithionite (54) Thus, inhibition of cytochrome P450 reduction was identified as at least one mechanism of inhibition. We investigated whether anti-$P450_{lpr}$ could inhibit house fly reductase mediated reduction of cytochrome *c*. No statistically significant inhibition of cytochrome *c* reduction was observed (54).

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We also examined the possibility that inhibition of P450 monooxygenase activity by anti-P450$_{lpr}$ was due to a non-specific effect such as disruption of the microsomes or protease contamination. Since anti-P450$_{lpr}$ does not recognize rat liver P450s (45), we challenged antiserum-treated 3-methylcholanthrene induced rat liver microsomes with ethoxyresorufin, phenobarbital induced microsomes with pentoxyresorufin, and both with ethoxycoumarin. These substrates are considered diagnostic for the P450s induced in these microsomes (60, 61). No inhibition of rat cytochrome P450 monooxygenases by anti-P450$_{lpr}$ was seen (54). Thus, anti-P450$_{lpr}$ did not show any non-antibody inhibition in rat liver microsomes or any antibody dependent inhibition of rat P450s. These results suggest inhibition of P450 monooxygenases by anti-P450$_{lpr}$ is due at least in part to interference of interaction between reductase and cytochrome P450. IgGs are large (150,000 Daltons) flexible molecules, and inhibition of reduction could be due to simple steric hindrance or disruption of the reductase-cytochrome P450 binding by disrupting the orientation of the cytochrome P450 in the membrane or alteration of cytochrome P450 tertiary structure. A general mechanism like this may be a common method of inhibition by anti-P450 polyclonals since all polyclonals are inhibitory to the cytochrome P450 they are directed against (55).

## Comparison of P450$_{lpr}$ with Other Known P450s

P450$_{lpr}$ shares the ability to hydroxylate benzo(a)pyrene with mammalian cytochrome P450s such as IA1 (54). However, the N-terminal sequence of P450$_{lpr}$ does not share any homology with rat P450 IA1, nor does anti-P450$_{lpr}$ recognize rodent P450 IA1 (54). Arguments concerning evolutionary distance between insect and mammal P450s, and immunological studies of insect and mammalian cytochrome P450 reductase, predict that insect cytochrome P450 would show homology at the catalytic site, but not in other parts of the molecule compared to mammalian cytochrome P450 (62). The present data supports this hypothesis, in the sense that P450$_{lpr}$ shares a catalytic preference for benzo(a)pyrene with P450 IA1, but homology of the whole molecule is not evident.

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## Chapter 3

# Expression of the Cytochrome P450 Gene *CYP6A1* in the Housefly, *Musca domestica*

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Cytochrome P450 enzymes play a major role in the metabolic resistance of insects to a variety of insecticides. A house fly P450 gene named *CYP6A1* has a high level of constitutive expression in an insecticide-resistant strain (Rutgers) when compared to a susceptible strain (sbo), suggesting that it may be involved in metabolic resistance. We have compared the levels of *CYP6A1* mRNA in fifteen strains of the house fly and show that expression of the *CYP6A1* gene is not always elevated in strains (such as R-Fc) known to possess a metabolic resistance mechanism. The *CYP6A1* gene is rapidly inducible by phenobarbital given in the drinking water of adult flies of both resistant and susceptible flies. Other chemicals such as DDT and dieldrin, known to induce P450 activities in house flies, did not induce *CYP6A1*. These results show that metabolic resistance in the house fly must involve the regulation of expression of more than one P450 gene, and that the induction mechanism of P450 genes by xenobiotics, including insecticides, is selective for some P450 genes.

The house fly, *Musca domestica*, has been a favorite insect in studies of insecticide resistance and in particular in studies of metabolic resistance mediated by cytochrome P450 enzymes (mixed-function oxidases). The major types of physiological / biochemical mechanisms of insecticide resistance (reduced penetration, reduced target site sensitivity, increased metabolism) have all been documented in one or more strains of the house fly (*1*). For instance, reduced penetration has been studied in organotin- and cyclodiene-resistant strains, and there are many examples of decreased target site sensitivity such as altered acetylcholinesterase, effects on sodium channels (in *kdr* and super-*kdr* flies, see chapter by Osborne and Pepper), or on $GABA_A$ receptors-chloride channels (in the case of cyclodiene resistance, see chapter by ffrench-Constant and Roush). Increased levels of DDTase and other glutathione transferases, esterases or P450 enzymes have all been documented in the house fly. In many cases, biochemical studies are complemented by genetic data characterizing inheritance patterns of resistance mechanisms(*2,3*). In view of this wealth of information on the biochemistry and genetics of resistance in the house fly, it would seem logical to continue to use this species as a "model" for resistance in other insect pests.

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The techniques of molecular biology are being increasingly brought to bear on the study of insecticide resistance in *Musca domestica*.. A glutathione S-transferase gene that is overexpressed in the Cornell-R strain was recently cloned and sequenced (*4*), and preliminary evidence indicates that more than one glutathione S-transferase gene is overexpressed in this strain, thus confirming the biochemical and genetic evidence provided by Ottea and Plapp (*5*). Recently, the amino acid sequence of a house fly cytochrome P450 called CYP6A1 was obtained from the sequence of its cloned cDNA (*6*). Although the catalytic activity of this P450 is still unknown (i.e. the substrates that can be metabolized by this P450 have not been identified), it is possible that CYP6A1 is contributing to metabolic resistance. Indeed, the *CYP6A1* gene was isolated by antibody selection of a cDNA library prepared from phenobarbital-treated Rutgers house flies. It is therefore likely to represent a major form of P450 in microsomes from this strain. Furthermore, it was shown that *CYP6A1* is constitutively overexpressed in the Rutgers strain when compared to the susceptible marker strain sbo (our unpublished data). The higher levels of *CYP6A1* mRNA in the Rutgers strain are not the result of massive amplification of the *CYP6A1* gene, as evidenced by Southern blot and dilution dot blot hybridization of genomic DNA. The developmental pattern of expression of the house fly *CYP6A1* gene closely resembles the pattern of many P450 activities in the house fly, with low levels in eggs, increasing levels in larvae with a maximum in day 4 larvae, a precipitous decline in pupae and a rise in adults, with no major difference between males and females. Throughout development, the levels of *CYP6A1* mRNA are significantly higher in Rutgers flies than in sbo flies, and this difference in mRNA levels is genetically controlled in part by chromosome II. Indeed, sbo larvae or adults carrying just one copy of chromosome II from the Rutgers strain show elevated levels of *CYP6A1* mRNA (our unpublished data). The overexpression of the *CYP6A1* gene may therefore represent a new type of molecular mechanism for resistance, different from the gene amplification seen for esterases in aphids and mosquitoes (*7*) and from the point mutation(s) apparently responsible for the insensitivity of acetylcholinesterases to organophosphorus and carbamate insecticides (*8*).

We report here the results of a screen of fifteen house fly strains which show that, although most insecticide-resistant strains have a higher expression of *CYP6A1* than most insecticide-susceptible strains, there is not always a correlation between high *CYP6A1* expression and resistance. We also document the rapid induction of *CYP6A1* by phenobarbital, and show that the *CYP6A1* gene is not inducible by several other known inducers of P450 in the house fly. We interpret the strain survey and induction results as implying the existence of multiple P450 genes and discuss some implications of these results in the study of insecticide resistance.

## Materials and Methods

**House fly strains**. The Diazinon-R "Rutgers" strain is a multi-resistant strain with elevated levels of P450 (*9*). This strain was maintained under rigorous diazinon selection. TriChE was derived from the Cornell resistant strain (*5*) crossed with a susceptible Cornell strain and sib-mated for several generations until target site (AChE) resistance was established. TriChE has no metabolic resistance to insecticides. Kdr-o is another target site resistant strain, with resistance to DDT confered by a gene on chromosome III (*kdr*). Baybo was derived from a Baygon-resistant japanese stock with a metabolic resistance gene on chromosome II. This strain carries recessive morphological mutant markers on chromosomes II (*bwb*) and V (*ocra eye*). PR car clw is derived from a resistant stock with altered aliesterase. The strain has slightly elevated levels of P450 activity, and carries recessive mutant markers *car* and *clw* on chromosome II. Penn is a methomyl-selected strain from a Pennsylvania horse barn, resistant to OPs, carbamates and dieldrin. It has target site resistance to carbamates and OPs (FWP, unpublished results) and may have elevated metabolic resistance. Tcar is a strain with elevated glutathione S-transferase activity. The Tcar flies are reported to be heterozygous for a metabolic resistance gene on chromosome II, and they carry the recessive mutant marker *car* on chromosome II (*5*). bwb;ge is a susceptible strain carrying mutant markers *bwb* and *ge* (green eye) on chromosome III. aabys is a susceptible strain carrying recessive mutations *ali-curve, aristapedia, bwb, yellow eye, snipwing* on chromosomes I, II, III,IV and V respectively (*10*). sbo similarly carries *stw, bwb* and *ocra* on chromosomes II, III and V respectively (*8*). S+ is a wild-type, susceptible strain derived from a single female of the Orlando-S strain. Orlando-DDT is a DDT/dieldrin resistant strain with target site resistance to DDT and pyrethroids (kdr) on chromosome III and target site resistance to cyclodienes on chromosome IV. In addition it has metabolic resistance to DDT on chromosome II and normal P450 activity (*11*). RFc is a wild type resistant stock of European origin with elevated P450 levels (*12*.) The LPR strain is a multiresistant strain that has very high pyrethroid resistance and elevated P450 levels (see chapter by Scott and Wheelock). Resistance in ASPR females is partly suppressible by piperonyl butoxide, thus implying P450-mediated detoxification as (one of) the resistance mechanism(s) in this pyrethroid-resistant strain (*13*).

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**RNA extraction.** Flies were flash-frozen in liquid nitrogen and stored at -80°C until use. Frozen abdomens were cut from other body parts over dry ice and were homogenized in 3 ml of ice-cold guanidine isothiocyanate (GIT) buffer. Homogenates were centrifuged to remove tissue debris, and the supernatant was layered on top of 5.7 M CsCl (*14*). The RNA was pelleted through CsCl in a Beckman 50.1 rotor at 32,000 rpm for 22-24 hours, and resuspended in 300 µl of 0.3 M sodium acetate, precipitated with ethanol and resuspended in diethylpyrocarbonate-treated water. Poly(A)$^+$-enriched RNA (mRNA) was isolated by oligo(dT)-cellulose chromatography (*14*). We typically obtained 15-25 µg mRNA from 15 abdomens of adult female flies.

**Northern and Dot hybridization procedures.** Northern blot hybridization was performed as described previously (*6*). For dot hybridization, poly (A)$^+$ RNA was applied in two-fold serial dilutions onto nitrocellulose using a 96-well Hybri-dot manifold (BRL). In each case, a minimum of two independent RNA isolates was analyzed. Two µg was the highest mRNA amount applied to the filter (see Fig. 3A). After application, the nitrocellulose filters were baked at 80°C *in vacuo* for 2 h. Prehybridization was carried out in 50% formamide, 5x SCC, 5x Denhardt's, 0.1% SDS, 0.005M EDTA, 0.05M sodium phosphate buffer pH 6.8 containing 50 µg/ml each of salmon sperm DNA and yeast tRNA for 6h at 42°C. Hybridization with nick-translated *CYP6A1* cDNA insert (*6*) was done in 50% formamide, 1x Denhardt's, 0.2% SDS, 0.005M EDTA, 0.02M sodium phosphate buffer pH6.8, containing 50 µg/ml each of salmon sperm DNA and yeast tRNA at 42°C for 22 hours. The filter was then washed sequentially in 2x SSC, 0.1% SDS and 0.5x SSC, 0.1% SDS at 27°C and analyzed by autoradiography. The uniformity of loading of the mRNA was verified by hybridization of the same filter with a nick-translated actin probe from *Drosophila* (DmA2). Determination of the relative *CYP6A1* mRNA levels was done either by scanning the autoradiograms with an LKB densitometer, or by visual comparison, using the 2-fold dilution series to estimate differences in dot intensity. The relative numerical values were transformed to their base 2 logarithms and normalized with respect to base 2 logarithms of the actin signals. An analysis of variance (ANOVA) was then performed on the transformed and normalized values using the SAS general linear model (GLM). Comparisons of means was done through Duncan's multiple range test.

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## Results

**Comparison of various house fly strains.** The levels of *CYP6A1* mRNA were compared in thirteen different house fly strains. Nine strains were known to be resistant to one or more classes of insecticides, and several mechanisms of resistance, including metabolic resistance are represented in these strains (see Materials and Methods for a full description of the strains). Four strains in our survey were susceptible strains, either wild type or carrying visible mutant markers. Figure 1 shows that there is a wide range in the expression of the *CYP6A1* gene among different house fly strains. Highest levels of *CYP6A1* mRNA were observed in the Rutgers strain, and only two resistant strains (Orlando-DDT and Fc) had levels similar to those of our reference sbo strain. Interestingly, two strains which are resistant in virtue of an altered cholinesterase (TriChE) or knock-down resistance mechanism (kdr-o), and are not believed to have a metabolic resistance mechanism have much higher *CYP6A1* mRNA levels than the four susceptible strains of our survey. We conclude that there is a considerable genetic variability in the expression of the *CYP6A1* gene in the house fly, and we need to consider the relationship between mRNA levels and protein levels carefully.

In addition to the 13 strains shown in figure 1, we also tested two pyrethroid-resistant strains, LPR and ASPR provided by Dr. J.G. Scott (Cornell University). Figure 2 shows that *CYP6A1* mRNA levels in the LPR strain are even higher than those observed in the Rutgers strain. However, neither males or females of the ASPR strain show mRNA levels higher than those found in the sbo strain. Although Northern blot analysis is not as quantitative a method as the dilution dot blots used to obtain the results of Figure 1, it is nonetheless clear that pyrethroid resistance in the ASPR strain is not caused by increased expression of the *CYP6A1* gene. The high expression of *CYP6A1* in the LPR strain suggested that CYP6A1 was involved in permethrin resistance. We therefore tested the toxicity of permethrin in a standard bioassay, in which adult flies are confined to pint jars coated with insecticide. Table I shows that Rutgers flies, although highly resistant to DDT and diazinon (about 100-fold), are still suceptible to permethrin. Thus, CYP6A1 probably does not contribute very much to the metabolism of permethrin in Rutgers flies, or to the resistance of LPR flies to permethrin.

**Table I. Toxicity of Permethrin to 3 Day-old Adult Female Flies**

| | Strain | | |
|---|---|---|---|
| | sbo | Rutgers | LPR |
| $LD_{50}$ (μg/jar) | 3.4 | 8.8 | 1669 |
| 95% C.L. | 3.0-3.7 | 7.9-9.9 | 1597-1744 |
| slope (SE) | 5.49 (0.42) | 3.41 (0.15) | 4.95 (0.23) |

**Time course of induction by phenobarbital.** Phenobarbital was added to the drinking water of adult flies, and the relative levels of *CYP6A1* mRNA were measured after various time intervals. Figure 3 shows the time course of phenobarbital induction in the Rutgers strain. A statistically significant increase in

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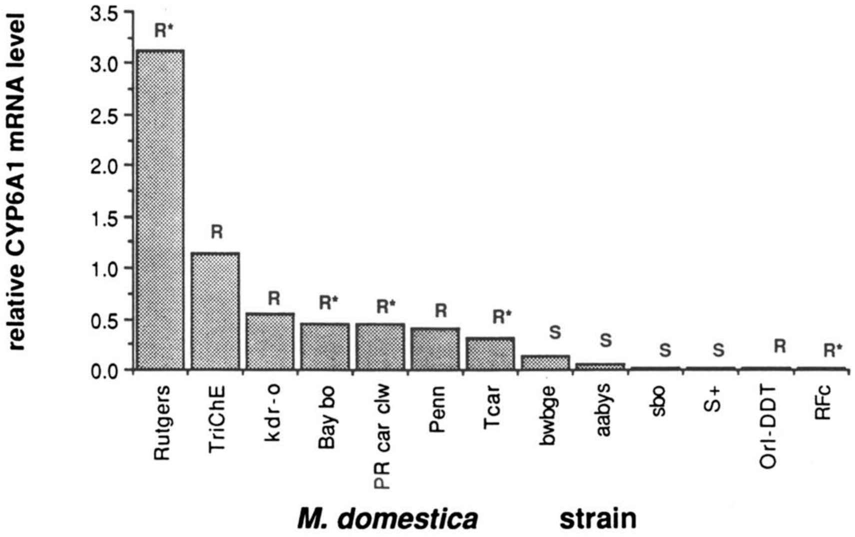

Figure 1. *CYP6A1* mRNA levels in 13 strains of *Musca domestica*. Poly(A)$^+$ RNA from 5 day-old adult females was hybridized to nick-translated *CYP6A1* cDNA probe. See Materials and Methods for a full description of each strain. R and S indicate insecticide resistance or susceptibility in that strain. R* indicates that metabolic resistance associated with P450 activity has been reported in that strain.

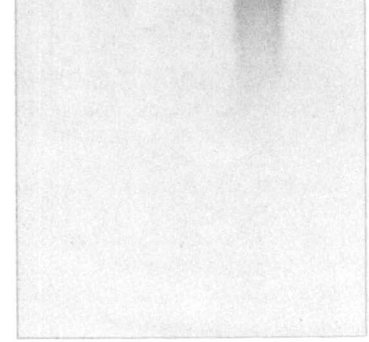

Figure 2. Northern blot analysis of poly(A)$^+$ RNA extracted from 3 day-old adult flies of the sbo, Rutgers, ASPR and LPR strains. Two µg mRNA was loaded in each lane. The size of the transcript from Rutgers and LPR strain is 1.9 kb (arrow).

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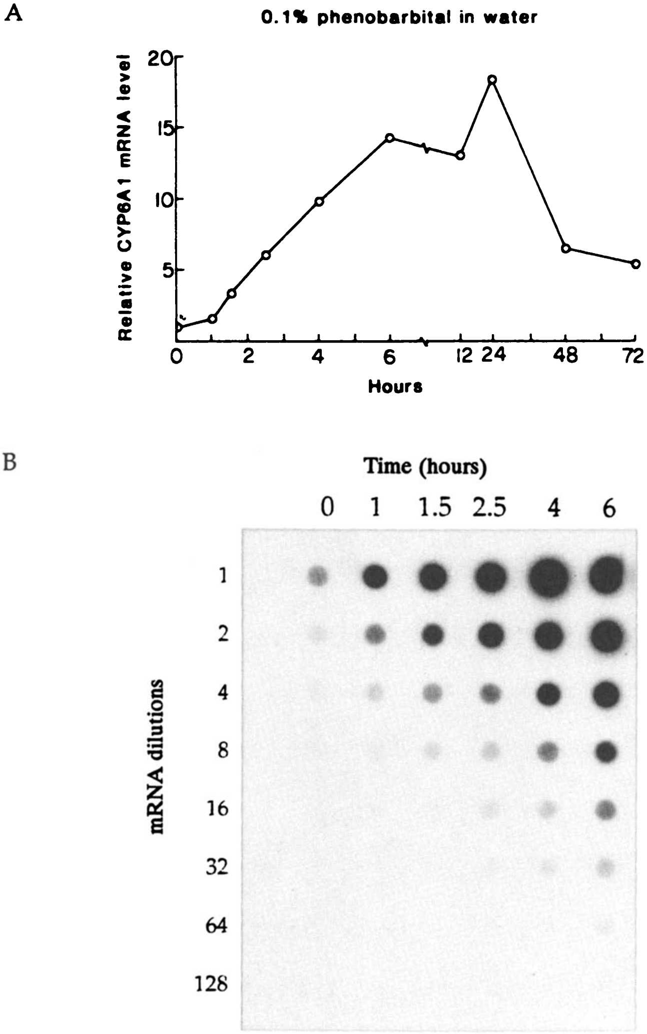

**Figure 3.** Time course of phenobarbital induction of the *CYP6A1* gene in 3 day-old adult female house flies of the Rutgers strain. Phenobarbital was added to the drinking water of adult females at a concentration of 0.1%, and 2 to 4 RNA samples were extracted at the times indicated. Panel A: Relative mRNA levels plotted versus induction time. Each point represents the mean RNA level (n= 2-4) for the treated flies divided by the RNA level of control flies of the same age. Panel B: Example of RNA dot blot for one experimental series. Two-fold serial dilutions of poly(A)$^+$ RNA starting from 2 µg (row "1") to 15.6 ng (row "128") were loaded onto nitrocellulose and hybridized to nick-translated *CYP6A1* cDNA probe.

mRNA levels was observed after 60 min, and reached near-maximal levels after 6 hours. Essentially similar results were obtained with sbo flies. Interestingly, flies kept on phenobarbital showed a decrease in *CYP6A1* mRNA levels 48 and 72 hours after the start of the experiment, indicating that high levels of induction were not maintained beyond 24 hours. A very similar pattern was observed for total P450 (reduced CO-difference spectrum) and for several P450 enzyme activities (*15*).

**Phenobarbital dose-response.** Adult female flies of the sbo and Rutgers strains were given phenobarbital in their drinking water at concentrations varying from 0 to 0.3%. A significant increase in *CYP6A1* mRNA was seen at a dose of 0.01% in both strains. Maximal induction was difficult to assess because significant mortality occurred at the highest doses, especially in sbo flies (about 30% mortality at 0.1%phenobarbital). When measured at 12 hours after addition of 0.1% phenobarbital to the drinking water, Rutgers flies were induced approximately 22-fold and sbo flies were induced about 100-fold. The maximum level of induced expression of *CYP6A1* therefore is approximately the same in the two strains. Addition of 0.05% phenobarbital to the larval food also leads to an induction of *CYP6A1* in both strains. Northern blot analysis did not reveal any difference in the size of the *CYP6A1* transcript between larvae, adults, induced and controls of either strain (results not shown).

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**Effect of other P450 inducers on *CYP6A1* mRNA levels.** Fifty 3 day-old adult female flies of the Rutgers strain were exposed to various potential inducers for a period of 24 hours. Preliminary experiments determined the highest concentrations at which these chemicals caused less than 30% mortality, and the chemicals were chosen because they had been shown to induce P450 enzymes in the house fly. ß-naphthoflavone (1%), piperonyl butoxide (1%) and naphthalene (0.1%) were administered by mixing these chemicals in the adult diet. Mortality at the stated concentration was 6, 12 and 18% respectively. Ethanol was added to the drinking water at a concentration of 10%, which caused 14% mortality. DDT and dieldrin were coated on the surface of pint jars at 1 mg/jar and 1 µg/jar respectively, causing 8 and 6% mortality. Control flies had mortality rates not higher than 2%.

Figure 4 shows that phenobarbital was by far the best inducer, causing an 18-fold increase in *CYP6A1* mRNA levels. Piperonyl butoxide caused a 4-fold increase, and ethanol caused a marginally significant doubling of mRNA levels. None of the other inducers tested caused an elevation of mRNA levels. From this experiment we conclude that the enzyme activities induced by naphthalene, dieldrin, DDT, and ß-naphthoflavone must involve P450 genes different from *CYP6A1*.

**Tissue distribution of *CYP6A1* expresssion.** Fifteen 4-day-old larvae were dissected into three compartments: fat body, gut (without Malpighian tubules) and "carcass" i.e. mostly integument and muscle. RNA was extracted by the hot phenol method and 15 µg total RNA from each sample was separated on an agarose/ formaldehyde gel. Northern blot analysis using *CYP6A1* cDNA as probe revealed that the highest level of message was located in the gut, with approx. 15-fold lower levels in the fat body. The transcript size was identical in both tissues (1.9 kb). Little or no message could be detected in the "carcass' compartment. This preliminary experiment indicates that *CYP6A1* is selectively expressed in certain fly tissues, especially the gut. Previous work on the distribution of aldrin epoxidase activity in house fly larvae (*16*) had shown considerable activity in the fat body of larvae. Clearly, much work remains to be done to correlate mRNA levels in a particular tissue with actual levels of CYP6A1 protein. This will be possible once specific antibodies to CYP6A1 are available.

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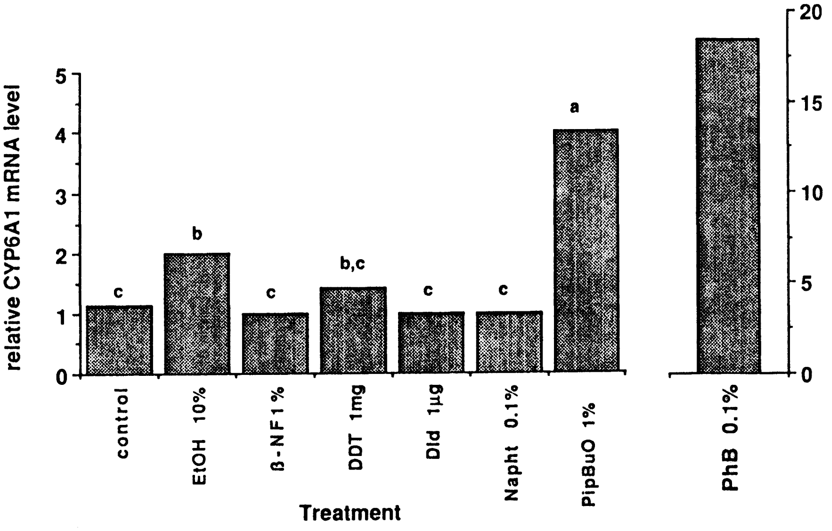

Figure 4. Effect of various chemicals on the level of *CYP6A1* mRNA in adult female flies of the Rutgers strain. EtOH: ethanol; ß-NF: ß-naphthoflavone; Dld: dieldrin; Napht: naphthalene; PipBuO: piperonyl butoxide; PhB: phenobarbital. The mode of administration and dosage of these chemicals is explained in the text. Relative levels of mRNA were estimated after dot-blot hybridization of 0.5, 1 and 2 µg of poly(A)$^+$ RNA from two samples of fifteen abdomens and three samples of 15 abdomens from control (uninduced) flies. Bars identified by the same letter are not statistically different.

## Discussion

Schonbrod *et al.*(*11*) noted in a 1968 survey of fourteen house fly strains that there was no simple relationship between insecticide resistance and levels of microsomal oxidases (they measured naphthalene hydroxylation and aldrin epoxidation). The involvement of more than one enzyme was suggested at the time to explain the lack of cross tolerance between a carbamate resistant strain and a naphthalene resistant strain. The present results agree with this explanation at the molecular level. It is very likely that the selection pressure of insecticides acted on different P450 genes on different occasions. Thus, the multiplicity of P450 genes in the house fly is now reflected in a multiplicity of P450-mediated metabolic resistance cases. In some strains an increased expression of P450 gene *A* would result in resistance, while in other strains, expression of P450 genes *B* and *C* might be increased. For example, both the Fc strain and the Rutgers strain are known to have elevated P450 levels (*17*), yet *CYP6A1* mRNA levels are elevated in the Rutgers strain, but not in the Fc strain (Figure 1). We imply that another P450 gene must be overexpressed in the Fc strain and account for resistance. Whenever resistance is associated with a large increase in the total P450 level (measured by the reduced CO-difference spectrum), the P450 gene(s) responsible for resistance is likely to be a major form of P450 in the reference susceptible strain. For instance, Wheelock and Scott (*18*) have shown that one P450 enzyme called P450lpr (or a few immunologically indistinguishable P450s with similar molecular weight and chromatographic properties) is increased from about 7% of the total P450 in a susceptible strain to about 67% in the pyrethroid-resistant strain LPR (see also Scott and Wheelock, this volume). This increase in P450lpr is associated with very high resistance to pyrethroids and a four-fold increase in total P450 level (*18*). Our results also suggest that *CYP6A1* is not involved in pyrethroid resistance in the ASPR and LPR strains. Because at least 7 chromatographically distinct P450 forms are found in adult house fly microsomes (*19*), and because the number of identifed P450 genes in the rat is at least 40, it is likely that the multiplicity of P450 genes in any insect species will continue to be a major object of investigation.

The studies on *CYP6A1* induction provide another line of evidence for the multiplicity of P450 genes involved in insecticide resistance. Phenobarbital (*15,20*), DDT (*21*), dieldrin (*21*), naphthalene (*20*), ß-naphthoflavone (22) and piperonyl butoxide (*23*) induce several P450 enzyme activities and some of these inducers may cause, depending on the strain, a slight decrease in the acute toxicity of insecticides (*20,24*). Although we have not yet tested PCBs, alpha-pinene, JH analogs or clofibrate all of which have been reported to induce P450 or P450 activities in the house fly, our screen of inducers shows a remarkable specificity of *CYP6A1* induction. In addition to phenobarbital, only piperonyl butoxide appears to be an inducer, and the molecular mechanism of induction by this methylenedioxyphenyl inhibitor of P450 enzymes may be quite different than that of phenobarbital, because of its initial inhibitory effect on P450 activities. If naphthalene, for instance, can double the amount of P450 and double the $LD_{50}$ of Baygon in the Fc strain (*20*), then CYP6A1 cannot be playing an important part in metabolic resistance of the Fc strain. Indeed, the *CYP6A1* gene has a low level of expression in the Fc strain, and is not inducible by naphthalene. The time course of induction by phenobarbital shows a very rapid increase in mRNA levels, and Brattsten et al. (*25*) have shown a very rapid induction by pentamethylbenzene in the southern armyworm. Also of interest is the decline in mRNA levels after 24 hours, which has been observed before (*15, 20*) and which suggests that a feedback loop turns off the *CYP6A1* gene even in the continued presence of the inducer.

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## Chapter 4

# Regulation of the Gene for *Drosophila* P450–B1, a P450 Isozyme Associated with Insecticide Resistance

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A structural change in the gene is involved in regulating the expression of *Drosophila* P450-B1, an insecticide resistance-associated P450 isozyme. The P450-B1 genes from a susceptible, 91-C, and a resistant, 91-R, strain were cloned and characterized. The gene of 91-C contains a solitary long terminal repeat (LTR) of transposable element *17.6* in its 3' untranslated region. It is absent from the gene of 91-R. It is postulated that an unstable, chimeric P450-B1 mRNA is made in the susceptible fly as a consequence of the presence of the LTR. Thus, the LTR is acting as a negative regulatory element to drastically reduce the level of P450-B1 in susceptible flies.

The adverse socioeconomic effects of insect pests are well known. Our capacity to control insect populations is seriously compromised by their ability to develop resistance to insecticides. Although the physiological and biochemical processes by which insects resist the toxic effects of insecticides are known, the molecular mechanisms by which these processes are regulated are not well understood. A better understanding of resistance at the molecular level could possibly lead to development of strategies to prevent or reverse the development of resistance.

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Metabolic detoxification of insecticides by cytochrome P450 monooxygenases is a major mechanism of resistance (*1-3*). The amounts of total P450 and/or specific isozymes are generally greater in resistant insects than in their susceptible counterparts (*4-21*). How the levels of P450 isozymes are regulated is not known. *Drosophila melanogaster* is a useful model system for studying the mechanisms by which resistance-associated P450 genes are regulated (*22*). Studies from our laboratory in this area of research are reviewed in this chapter.

## Characterization of Resistance-Associated P450s in Drosophila

Earlier studies in this laboratory showed that the amount of nitrosodimethylamine demethylase (NDMA-d), a microsomal, P450-dependent activity in *Drosophila*, is strain dependent (*20,23,24*). Total P450 content is also greater in NDMA-d-positive strains (*16,20,24*). Because of the large differences in P450 content among strains, microsomal proteins from Hikone-R (BG) and Oregon-R, prototype strains for high and low NDMA-d activity and P450 content, respectively, were analyzed by SDS-PAGE. A single heme-containing band (band A, ~ 59.3 kDa) is present in both Oregon-R and Hikone-R (BG) and in all of more than 20 other strains tested (*24*). An additional band (band B, ~ 55.8 kDa) is present in Hikone-R (BG) and only in a few other strains. Because bands A and B contain the only microsomal proteins that stain for heme (*24*) and are the only bands to be selectively enriched by standard P450 purification methods (*25*), it is likely that most, if not all, of the P450 isozymes in *Drosophila* are contained in these two bands. Various lines of evidence indirectly indicate that each of these bands contain multiple isozymes. These have been discussed in previous publications (*20,26*). The P450 isozymes in bands A and B are designated P450-A and P450-B, respectively (*20*). The presence of band B is positively correlated with NDMA-d activity and resistance to phenylurea (*20,24*). Genetic and biochemical analyses indicate that the genes required for P450-B expression, and NDMA-d activity, are located on chromosome II at, or near, a major resistance locus (*20*). Resistance to DDT, phenylurea, parathion, and carbaryl have been mapped to this locus (*27-30*). P450-B is probably the product of genes at that locus.

Strain dependent expression of the P450-B subset of P450 isozymes and the association of P450-B with NDMA-d activity and insecticide resistance makes *Drosophila* a useful system with which to study regulation of resistance-associated P450 genes at the molecular level.

## Monoclonal Antibodies to Isozymes of P450-A and P450-B

Further studies on the molecular mechanisms that regulate P450-B expression in *Drosophila* required that we generate probes with which to

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identify and quantify specific P450 isozymes. Traditional chromatographic methods were used to partially purify components of the two P450 subsets (*25*). A preparation containing a mixture of P450-A (40%) and P450-B (60%) was used as antigen to produce a number of monoclonal antibodies (MoAbs). Two of these, 13-2e and 8-1d, were extensively characterized and shown to react with isozymes of the P450-A and P450-B subsets, respectively (*26*). We have designated these immunoreactive isozymes as P450-A1 and P450-B1. The quantities of total P450-A and P450-B, as well as immunoreactive P450-A1 and P450-B1, were estimated from densitometric scans of SDS-PAGE gels and Western blots, respectively, in pairs of susceptible/resistant strains, i.e., Oregon-R/Hikone-R (BG) and 91-C/91-R (*26*). [Hikone-R was collected as a DDT-resistant strain from the field in 1952 (*27*). It is also resistant to various other insecticides including phenylurea, parathion and carbaryl (*28-30*). We find Hikone-R to be significantly more resistant to phenylurea and malathion than Oregon-R (*20,26*). Strain 91-R was selected from its control progenitor stock, 91-C, by exposure to DDT (*31*). In the mid 1960's, 91-R was about 70 times more resistant than 91-C to DDT and its resistance has continued to increase (*32*). Strain 91-R is also about 100 times more resistant than 91-C to malathion (*26*). Evidence for *Drosophila* P450-dependent metabolism of both malathion and DDT has been reported and these activities are higher in resistant than in susceptible strains (*19,33*).] The pattern of P450 isozyme expression was very similar in the two pairs. Total P450-A and P450-A1 levels were both somewhat less in the resistant strains, 20% and 50%, respectively. The amount of total P450-B, on the other hand, was estimated to be 50-100 times higher in the resistant strains. The amounts of P450-B1 were 10-20 times greater in the resistant strains than in the susceptible ones (*26*). To summarize, in susceptible strains the amounts of total P450-B and immunoreactive P450-B1 are barely detectable while in resistant strains they are present in much greater amounts which are comparable to those of P450-A and P450-A1 in either strain.

## Molecular Analyses of P450-B1 Gene Expression

MoAbs to P450-B1 provided us with a specific reagent with which to study the mechanisms by which expression of a resistance-associated P450 isozyme is regulated at the molecular level. The structure and function of P450-A1 is of interest in its own right; however, we are currently using P450-A1-specific probes, i.e., MoAbs and cDNA clones, as controls for our studies of P450-B1 gene regulation. Because strains 91-C and 91-R express P450-A and P450-B, as well as P450-A1 and P450-B1, quantitatively like Oregon-R and Hikone-R (BG) and because they are presumably isogenic except at resistance-associated loci, they were chosen for use in these studies (*26,31*).

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**Cloning and Characterization of P450-A1 and P450-B1 cDNAs.** A cDNA library was made from poly A$^+$ RNA isolated from the 91-R strain. P450-A1- and P450-B1-specific cDNA clones were identified and isolated by immunoscreening of the λ gt 11 (BRL) library. The largest P450-A1-specific clone was not full length and appears to contain only about 50% of the coding sequence. Several overlapping P450-B1-specific clones were obtained. A single clone, 1658 bp, contained an open reading frame of 1521 nucleotides corresponding to a protein of 507 amino acids. Two other clones provided an additional 31 bp, terminating in poly A stretches, for a total cDNA length of 1689 bp. The deduced 507 amino acid sequence of P450-B1 corresponds to a molecular mass of 58,835 daltons. This mass is different from the 55.8 kDa mass estimated from SDS-PAGE for P450-B, but is consistent with the fact that the molecular masses of P450 isozymes estimated by gel electrophoresis are generally lower than those determined from amino acid sequence (*34*). The deduced amino acid sequence of P450-B1 shares 49% positional identity with the house fly CYP6A1 and therefore shares several of the conserved amino acid sequences of P450 isozymes generally and of family 3 P450 isozymes specifically (*35,36*). Among the 23 amino acids around the putative heme-binding cysteine at position 452, i.e., F G D G P R N C I G M R F G Q M Q A R I G L A, 20 are indentical to those around the analagous position in CYP6A1 (*35*). The scientific name CYP6A2 has been assigned to P450-B1 (D. W. Nebert, personal communication).

**P450-B1 Gene Expression and Organization in Strains 91-C and 91-R.** Unique restriction fragments of P450-A1 and P450-B1 cDNAs were used as probes to measure the steady state levels of P450-A1 and P450-B1 mRNAs in strains 91-C and 91-R by Northern blot analysis (*37*). The same fragments were used to probe Southern blots for evidence of structural gene changes in the two strains.

There was 20-30 times more P450-B1 mRNA in 91-R than in 91-C (*37*). This increase is consistent with the approximate 20 fold increase in the amount of P450-B1 protein in 91-R relative to 91-C (*26*). These data indicate that there is a direct relationship between the amounts of P450-B1 mRNA and protein, and that a post-translational regulatory mechanism, involving P450-B1 stability, is not operative in these strains. The amount of P450-A1 mRNA was essentially the same in both strains and, as expected based on the relative sizes of the proteins, was larger than P450-B1 mRNA. The amounts of P450-A1 mRNA in 91-C and 91-R are similar, within a factor of two or so, to that of P450-B1 mRNA in 91-R (*37*). This is consistent with the relative amounts of the corresponding proteins in the two strains (*26*). These correlations indicate that the P450-B1 gene might be down-regulated in 91-C rather than being up-regulated in 91-R. An additional feature of the small amount of P450-B1 mRNA in 91-C is its size which is significantly larger than that in 91-R (*37*). This mRNA size difference could be indicative of a structural difference in the P450-B1 gene of the two strains.

Results of Southern blot analyses of the P450-B1 gene of 91-C and 91-R were consistent with there being a structural difference in the gene of the two strains. Single, unique P450-B1-specific fragments, in the size range of 0.6-25 kbp, were produced by digestion with *Bam*HI, *EcoR*I, *Hind*III, *Pst*I or *Sst*I. With three of these, *Bam*HI, *EcoR*I, and *Pst*I, restriction fragment length polymorphisms (RFLPs) were observed between the two strains. For example, the *EcoR*I fragment in 91-C, ~ 6.3 kbp, was about 400 bp longer than that in 91-R. Rehybridization of the same blot with the P450-A1 probe showed unique, P450-A1-specific restriction enzyme fragments and there was no evidence for RFLPs in the P450-A1 genes of the two strains. These analyses showed no evidence for relatedness between the P450-A1 and P450-B1 genes. Furthermore, neither Northern nor Southern blot analyses gave any indication that either gene is a part of a family of related genes. Hybridization intensities indicated that the P450-B1 gene is not amplified in the resistant 91-R strain, unlike the esterase genes involved in resistance to organophosphates in aphids and mosquitoes (*38,39*).

**P450-B1 Gene Cloning and Characterization.** In order to determine the structural difference(s) in the P450-B1 genes of 91-C and 91-R, the Lambda GEM-11 genomic cloning vector (Promega) was used to clone the gene from the two strains. Selected 91-C clones yielded the ~ 6.3 kbp *E. coli* fragment that was observed in the Southern analysis. Likewise, an ~ 5.9 kbp *EcoR*I fragment was isolated from 91-R genomic clones. These *EcoR*I fragments were cloned into the plasmid pGEM-7Z (Promega) for analysis. Restriction maps of these clones are shown in Figure 1. By comparison with the cDNA restriction map it was indicated that the coding sequence of the P450-B1 gene is uninterrupted, i.e., is intronless. However, the possible presence of very small introns has not been rigorously excluded and will require sequencing the coding region of the genomic clones. Comparison of the restriction maps of the 91-C and 91-R gene clones showed the major structural difference to be located at the 3' end of the P450-B1 gene of 91-C. A DNA sequence of about 500 bp, at positions 2536-3054, was present in the gene of 91-C, but was absent in the 91-R gene. A landmark of this sequence is an *Nsi*I site, at position 2694, which is absent in the gene of 91-R. This inserted sequence was identified as a long terminal repeat (LTR) of the *Drosophila* transposable element *17.6* (*40*). The site at which the LTR is inserted is downstream of the coding sequence of the gene, but is within the 3' untranslated region of the transcribed sequence. The precise location is 28 bp downstream of the translation termination codon and 48 bp upstream of the putative

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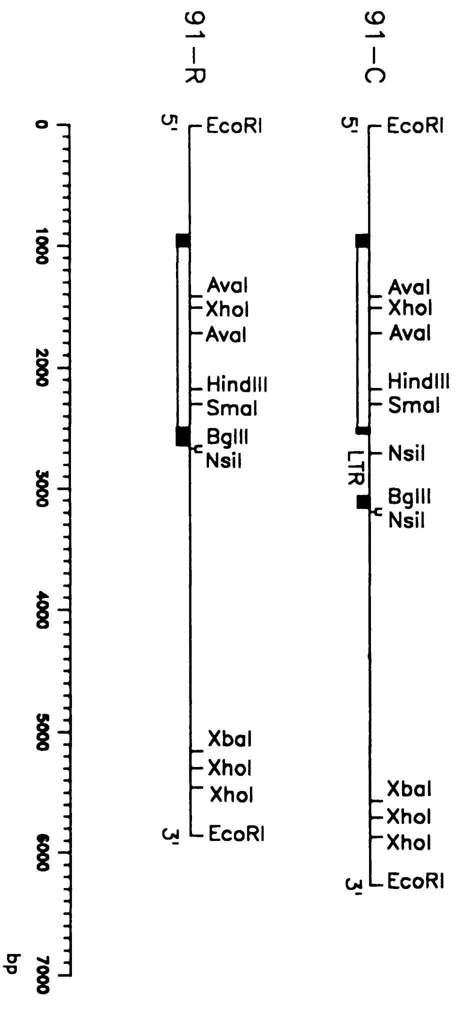
91-C
5'
EcoRI
AvaI
XhoI
AvaI
HindIII
SmaI
LTR
NsiI
BglII
NsiI
XbaI
XhoI
XhoI
EcoRI
3'
91-R
5'
EcoRI
AvaI
XhoI
AvaI
HindIII
SmaI
BglII
NsiI
XbaI
XhoI
XhoI
EcoRI
3'
0
1000
2000
3000
4000
5000
6000
7000
bp

**Figure 1. Restriction maps of the P450-B1 gene of *Drosophila* strains 91-C and 91-R.** The transcribed region of the gene is indicated by the box. The coding region is the open portion of the box. The closed portions of the box are the 5′ and 3′ untranslated regions of the gene. "LTR" indicates the position of the *17.6* LTR in the gene of 91-C.

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polyadenylation signal of the gene. The most obvious effect of the presence of the LTR is to move the polyadenylation signal and poly A site of the gene downstream by more than 500 bp. From this arrangement it can be predicted that the mRNA transcripts of the gene of 91-C would be chimeric, i.e., contain both P450-B1 gene and LTR sequences, and would be longer than those produced from the gene of 91-R. The results of Northern blot analysis of P450-B1 mRNA in 91-C and 91-R are consistent with this prediction. A summary of results of molecular analysis of the P450-B1 gene and its expression is given in Table I.

Table I. **Relative Expression and Organization of the P450-B1 Gene in Susceptible, 91-C, and Resistant, 91-R, Strains of *Drosophila***

| Parameter | Comparison |
|---|---|
| 1. Protein | ≥ 20 times more in 91-R[a] |
| 2. mRNA | ~ 20-30 times more in 91-R[a]; longer mRNA in 91-C |
| 3. Gene structure | 3′ untranslated region in 91-C interrupted by LTR of element *17.6*; absent in 91-R |

[a] The amounts of P450-B1 protein and mRNA in 91-R are approximately equal to those of P450-A1 protein and mRNA in either strain.

### Possible Mechanisms for Regulating P450-B1 Gene Expression in 91-C and 91-R

The phenomenon of insertional mutagenesis is well known. For examples, the *white-apricot* mutation in *Drosophila* and the *dilute* coat color mutation in DBA/2J mice are known to be caused by the insertion of transposable elements (*41,42*). In these cases the elements are apparently present in introns and cause premature termination or altered splicing of the gene transcripts. Interestingly, reversions at these loci have been shown to leave behind a solitary LTR which apparently is not mutagenic (*43,44*). However, in the case of the P450-B1 gene in 91-C, the presence of a solitary LTR may be sufficient to mutate the gene.

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When considering mechanisms by which the P450-B1 gene is regulated, it seems most reasonable to presume that the gene of 91-R is a normal constitutively expressed P450 gene. This is consistent with the relatively similar transcriptional activity of this gene as compared with the P450-A1 gene and the fact that we have no evidence for the inducibility of the P450-B1 gene. Thus it seems likely that the P450-B1 gene is down-regulated in the susceptible, 91-C strain as opposed to being up-regulated in the 91-R strain. At this point in our studies we believe that the presence of an LTR in the P450-B1 gene is directly responsible for the drastically reduced level of P450-B1 in the susceptible 91-C strain. We propose that the presence of the *17.6* LTR in the gene of 91-C results in synthesis of chimeric mRNA transcripts which, because of features of their structures, are unstable.

As indicated before, insertion of the LTR in the gene of 91-C effectively moves the normal polyadenylation signal about 500 bp downstream. The LTR itself contains two potential polyadenylation signals. Utilization of the first LTR, second LTR, or the normal polyadenylation signals would yield mRNA transcripts that are, respectively, about 120, 325 or 500 bp longer than those produced in 91-R. Transcripts that terminate by utilizing the normal signal are expected to be highly unstable due to the presence of three AUUUA sequences preceding that signal. These sequences have been shown to destabilize mRNAs (*45*). Alternatively, features of the chimeric transcripts could prevent their proper polyadenylation which in itself could render them unstable (*46,47*). The possibility of up-regulating elements, e.g., promotors or enhancers, being associated with the gene of 91-R has not been rigorously excluded, however so far none have been indicated.

**Model for Evolution of 91-C and 91-R P450-B1 Genotypes.** This study has identified genotypic differences in *Drosophila* strains which may well be important determinants of susceptible versus resistant phenotypes. Based upon known mechanisms of transposition of mobile genetic elements (*48*), one can speculate on the origin of the P450-B1 genotypes found in strains 91-C and 91-R. A cyclic model of the evolution of these genotypes is given in Figure 2. The 91-R genotype (structure I), i.e., lacking any part of the transposable element *17.6*, was probably the progenitor of the 91-C genotype (structure III). At some point in time a complete copy of element *17.6* was probably inserted at the 3' end of the P450-B1 gene to produce structure II by a sequence-specific mechanism (*49*). Flies with this genotype (structure II) would most likely be susceptible to insecticides like strain 91-C (structure III). At a later time, the structural genes of *17.6* and one LTR could have been deleted by homologous recombination. This would leave the single LTR in the gene (structure III) that we have found in strain 91-C. Because the P450-B1 gene is expressed at low levels, if at all,

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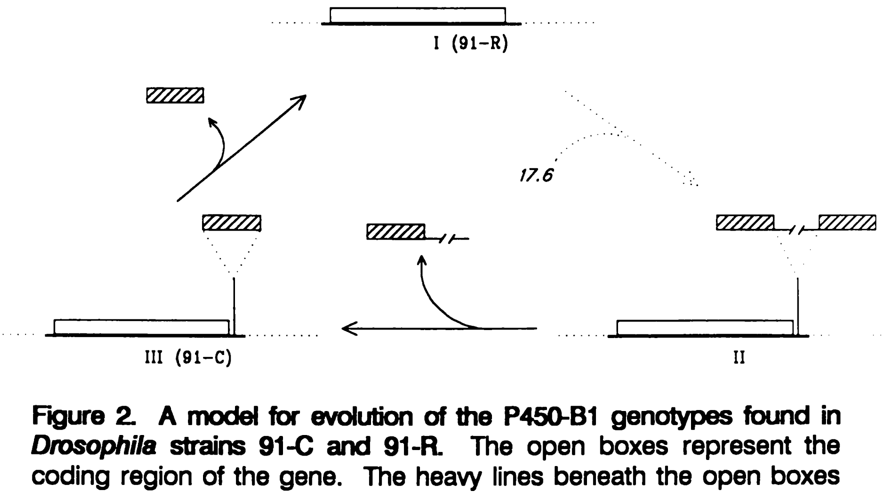

**Figure 2. A model for evolution of the P450-B1 genotypes found in *Drosophila* strains 91-C and 91-R.** The open boxes represent the coding region of the gene. The heavy lines beneath the open boxes represent the transcribed region of the gene. The shaded boxes represent the LTRs of transposable element *17.6* and flank the structural genes of *17.6*. Vertical lines indicate insertion sites for *17.6* (structure II) or its solitary LTR (structure III).

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in many laboratory strains of *Drosophila*, it must not be essential for survival of the organism under normal laboratory conditions. Thus, before introduction of insecticides, none of the gene structures shown in Figure 2 would have provided a selective survival advantage over the others. However, reversion to structure I, i.e., the 91-R genotype, by excision of the transposable element would confer a selective survival advantage to organisms exposed to DDT, and possibly to other insecticides. It remains to be determined whether insecticides simply select for spontaneous revertants or are involved in the reversion process.

## Summary of Molecular Studies on the P450-B1 Gene of *Drosophila*

To summarize our work, we have identified an electrophoretically-defined subset of *Drosophila* P450 isozymes, i.e., P450-B, that is associated with resistance to insecticides. The gene for an isozyme, P450-B1, from this subset was cloned and characterized. The gene in the resistant, 91-R strain is structurally different from the gene in the susceptible, 91-C strain but is not amplified. An LTR of transposable element *17.6* is present in the 3' untranslated region of the 91-C gene and is absent in the 91-R gene. We postulate that the presence of the LTR in the 91-C gene leads to the synthesis of chimeric mRNA transcripts that are unstable, accounting for the low level of P450-B1 in the susceptible strain. It remains to be determined whether this is the correct mechanism by which P450-B1 gene expression is regulated in strains 91-C and 91-R. And if it is, is it also operational in other *D. melanogaster* strains? Several other questions remain to be answered. What are the mechanisms that regulate genes for other resistance-associated isozymes of the P450-B subset? Will the mechanisms in *Drosophila* be representative of those involved in regulating resistance-associated P450 genes in other insects? Our future work will be directed toward precisely defining the mechanism of P450-B1 gene regulation and assessing the generality of such a mechanism for gene regulation.

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## Chapter 5

# Evolution of Glutathione *S*-Transferases Associated with Insecticide Resistance in *Drosophila*

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Glutathione S-transferases were analyzed in a malathion-resistant strain of *Drosophila*. We observed an approxiamtely three-fold elevation in total activity, that is associated with elevated levels of two GST isoforms, DmGST1 and DmGST2. Elevation of the former is associated with increases in specific mRNA. In the case of DmGST1, DNA sequence analysis of the gene that encodes the protein indicates the presence of a six nucleotide duplication in the coding region, resulting in the addition of two amino acids to the protein. The position of this substitution is distinct from the region of greatest sequence divergence among species, as shown by comparison of the coding sequence of *D. melanogaster* and *D. simulans*. Finally, we compared the sequences of these proteins to those of other published sequences. DmGST1 contains one region with similarity to bacterial and plant enzymes and another related to class alpha GST's from mammals. DmGST2 is most similar to enzymes of the pi class from both vertebrates and invertebrates. We conclude that in this case, evolved insecticide resistance has a polygenic basis, and involves changes in both structural and regulatory aspects of genes encoding detoxification proteins.

Evolved insecticide resistance is a phenomenon that is of obvious economic importance and provides an opportunity to observe the evolutionary process as it occurs in a finite time period. Evolution of resistance has been repeatedly observed in both field *(1-3)* and laboratory *(4,5)* populations, and in many cases it has been possible to associate changes in the structure or expression of particular gene products with the resistant phenotype. Two

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questions can thus be addressed. First, what are the physiological changes associated with adaptation to pesticide stress? Second, what are the molecular events that underly that adaptation? Data relating to these questions should be highly informative with respect to both pest management strategies and our understanding of the molecular basis of the evolutionary processes.

**The genetics of resistance.** The evolution of insecticide resistance can be rapid, suggesting the involvement of a small number of genes *(6,7)*. Furthermore, this rapid response to selection implies that allelic variation of those genes exists in populations, so that selection can alter the genetic makeup of the population without being dependent upon the occurrence of new mutations. Thus, to understand the process, we need to identify the responsible genes, and also characterize allelic variation of those genes in both unselected populations and in those that show an altered phenotype associated with resistance.

Two approaches can be taken to identify relevant genes. First, we can examine the structure or expression of gene products for which we have *a priori* expectations of involvement with resistance. These may include detoxification proteins (cytochromes P450; esterases; glutathione S-transferases) or target sites of insecticide action (acetylcholine esterases). Alternatively, we could employ screening strategies to obtain probes, either nucleic acid or antibody, that identify genes or gene products associated with resistance, and attempt to determine their function based upon sequence analysis. Once genes are identified, we then need to ask what molecular differences exist between alleles that contribute to resistance and those found in sensitive insects. Such differences could be ones that affect the primary structure of the gene product, its abundance, or both. Biochemical analyses have implicated each of these phenomena in evolved resistance *(8)*. With the advent of facile methods for the isolation and analysis of DNA sequences, it is now possible to precisely identify sequence changes associated with evolved resistance, and to place those differences within the context of sequence variation that exists within species or genera.

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**Insecticide resistance in *Drosophila*.** In this paper, we summarize our recent work on the expression and structure of glutathione S-transferases (GST's; E. C. 2.5.1.18) that show altered patterns of expression in a strain of *Drosophila melanogaster* that was selected for resistance to malathion *(9-11)*. Wilson *(12)* and ffrench-Constant and Roush *(13)* have presented the case for use of *Drosophila* as a model organism for analysis of the genetics of pesticide resistance. The key advantage of this organism is, of course, our ability to manipulate it genetically, and the existence of a germ line transformation system for establishment of causal relationships between particular genes and phenotypes *(14)*. In addition, selection for insecticide resistance has been successful *(9)*, and changes in structure or expression of particular genes, known to be associated with resistance in pest organisms, has been demonstrated *(15-21)*. One caveat regarding *Drosophila* is that typically levels of resistance obtained are lower than those of pest insects *(13)*; nevertheless, in those cases where changes associated with resistance have been analyzed at the biochemical or genetic level, it has been shown that they involve many of the same functions - detoxification proteins, target sites, etc. - associated with evolved resistance in other species *(15-21)*.

**Glutathione S-transferases in *Drosophila*** We have previously reported that most of the GST activity in adult *Drosophila* was found in a fraction froma glutathione agarose affinity column, that contains a single, heterodimeric protein *(22)*. Toung et al. *(23)* purified a protein from embryos, and obtained a cDNA clone, designated DmGST 1-1, and showed that active enzyme could be obtained following expression of that clone in *E. coli*. The gene encoding this protein is intronless, and is located at cytological position 87B on the right arm of chromosome III (24). We have subsequently determined that the sequence of that cDNA is identical to one specific for the small subunit of the adult protein, and it is a member of a small gene family (25) In addition, we have identified an additional GST-specific cDNA, designated DmGST2-1, based upon sequence similarity to knwon GST's *(26,27)*. This gene is situated on chromosome II at position 51F-52A *(28,29)*. Herein, we report that both of these proteins are present at elevated levels in a malathion-resistant laboratory strain of *Drosophila*, but that the mechanism responsible differs between the two. Furthermore, we have compared the sequence of the DmGST1 allele from sensitive and resistant flies, and find that the latter contains a six base duplication within the coding region, resulting in an insertion of two amino acids into the protein. Finally, we consider the structure of these two proteins from *Drosophila* in the context of the emerging picture of the sequence and structural organization of members of the GST family from a variety of species.

## Materials and Methods

**Reagents and Chemicals.** Restriction enzymes were obtained from either Boehringer Mannheim (Indianapolis, IN) or Promega Biotech (Madison, WI). Reagents and enzymes for the polymerase chain reaction were obtained from United States Biochemical Corp. (Cleveland, OH). $^{32}$P-deoxycytidine triphosphate (3000 Ci/mmol) and $^{35}$S-$\alpha$-thio- deoxyadenosine triphosphate (800 Ci/mmol) were purchased from New England Nuclear. All other chemicals and biochemicals were obtained from either Boehringer Mannheim or Sigma Chemical Co. (St. Louis, MO) and were of reagent grade or better.

***Drosophila* stocks.** Malathion-resistant strain IIID was derived from flies resulting from selection for malathion resistance by Singh and Morton *(9)*. Following 120 generations of selection, lines that were isogenic for each

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autosome were extracted and their contribution to resistance determined. Strain IIID is isogenic for chromosome III, and contains a major gene contributing to resistance at map position 3-55 *(10)*.

Other stocks employed include Canton-S, a standard laboratory strain that is sensitive to malathion, and Df(3R) kar27, is a deficiency for the region 87B5-87D6, and includes the DmGST1-1 structural locus *(24,25)*.

**GST purification and activity assay.** Assays of GST activity, employing 1-chloro, 2,4-dinitrobenzene (CDNB) and purification of GST were performed as described previously *(22, 28)*. Protein concentrations were determined by the method of Bradford *(29)*, using bovine serum albumin (BSA) as a standard.

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**Antibodies and Immunoblotting.** Polyclonal antibodies to the major GST in *Drosophila* were prepared by injection of GST, purified as described above, into rabbits, with blood collected by heart puncture, and serum prepared by standard methods. The antibody that was used to identify proteins overexpressed in strain IIID was similarly prepared. This antibody was intended to be used as a probe of cytochromes P450; hence microsomal proteins were fractionated by octylamino agarose and DEAE sepharose chromatography *(10)*. These proteins were then used as antigens for immunization. Protein preparation, Western blotting, and probing of filters were performed as described previously *(22,27)*.

**Nucleic acid preparation and hybridization.** Genomic DNA from *Drosophila* was prepared as described by Jowett *(30)*. RNA from adults was prepared using the guanidinium isothiocyanate procedure described by Chomczynski and Sacchi *(31)*. Plasmid DNA was purified by alkaline lysis and phenol-chloroform extraction, and cloned inserts isolated by agarose gel electrophoresis of appropriately digested plasmid DNA, followed by extraction of insert DNA from gel slices by the freeze-thaw method described in Sambrook et al. *(32)*. Northern blotting and probing were performed using standard methods *(26,33)*.

**The polymerase chain reaction (PCR) and DNA sequencing.** GST coding regions were amplified from genomic DNA, using the polymerase chain reaction *(34)*. Primer sequences employed for amplification of the entire coding region of DmGST1-1 were 5'-GGTTGACTTCTACTACCTGC - 3' and 5'-CGTGAATATCAGGCTTATTC - 3'; the first is identical to the sequence immediately downstream of the ATG initiation codon, while the second is complementary to a regions including the TAA termination codon *(23)*. PCR reactions were performed in a volume of 50 ul, using AmpliTaq DNA polymerase (U. S. Biochemical Corporation) according to manufacturers specifications, and 100 ng of genomic DNA as a template. PCR was performed in a BIOS Corporation BIOSycler automated thermocycler, with cycling parameters of 30 seconds each at 94 C, 55 C, and

72 C, for a total of thirty cycles. Direct sequencing of PCR products was performed by the method of Higuchi and Ochman *(35)*, using Sequenase (U. S. Biochemicals) as per manufacturer's specifications. Reactions were primed with various internal primers obtained from the published sequence of DmGST1-1; these sequences are available upon request.

**Computer analyses of sequence data.** General similarity searches were performed using FASTA as implemented in the GCG package of sequence analysis programs *(36,37)*, and searches for local similarity were performed using the program BLAST *(38,39)*. In both cases, the database searched was SWISS-PROT, version 18 *(40)*.

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## Results

**Elevation of GST activity in malathion-resistant flies.** Singh and Morton *(9)* reported and elevation in GST activity. We confirmed this observation by measurement of total activity towards CDNB in crude extracts of control (Canton S) and resistant (IIID) flies. As shown in Figure 1, the latter show a 2.5-fold elevation in activity in the resistant strain, relative to the control.

These results do not resolve the question as to which isoform of GST is responsible for the elevated enzymatic activity. In Figure 1, we show the effect of heterozygosity for a deficiency that includes the GST1-1 locus (25). The reduced activity indicates that this gene does in fact encode a protein responsible for much of normal adult activity. Figure 2 shows that DmGST1-1 encodes the small subunit of that protein, as evidenced by the reduced abundance of the protein detectable on Western blots. Figure 2a shows a Western blot of total proteins from both strains as well as deficiency heterozygotes, probed with anti-GST serum. The reduction in GST1 abundance in deficiency heterozygotes is expected, given the presence of only one allele of the gene in these flies. Panel 2b illustrates an SDS-PAGE separation of GST from Canton S and IIID adults, purified as described by Cochrane et al *(22)*. The higher abundance of the small subunit in the extracts of malathion-resistant flies is evident with both approaches. In contrast, there is no evident difference in abundance of the large subunit either associated with resistance or resulting from deficiency heterozygosity. Finally, densitometric scanning of the blot in Figure 2a demonstrated good correspondence between GST1-1 subunit abundance and total GST activity as shown in Figure 1 (data not shown).

We have used the antibody to obtain a clone specific for the small subunit; sequence analysis reveals that it is identical to the sequence DmGST1-1 of Toung et al. *(23,25)*. We used this clone as a probe of Northern blots of total RNA from Canton S and IIID adults. As shown in Figure 3, mRNA abundance is elevated in the resistant flies, to an extent comparable to the elevation of protein abundance (Figure 2). No difference in abundance of actin-specific mRNA's were detected in replicate blots,

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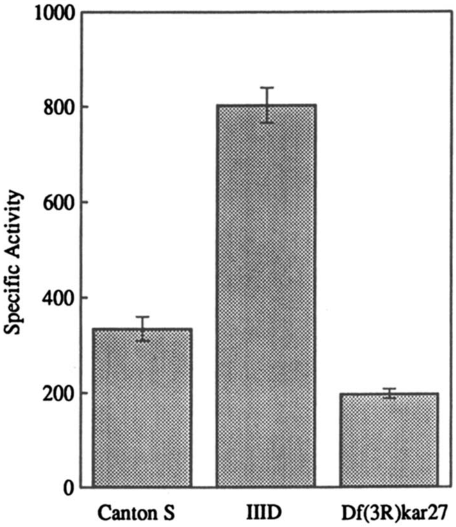

Figure 1. Total GST activity towards 1-chloro 2,4-dinitrobenzene in extracts of malathion-sensitive (Canton-S) and resistant (IIID) flies, as well as in flies heterozygous for a deficiency spanning the structural gene for DmGST1-1 (Df(3R) kar27/+). Activity units are in nmoles conjugate formed/min/mg protein. Values are means of six replicate assays; error bars represent the standard error of the mean.

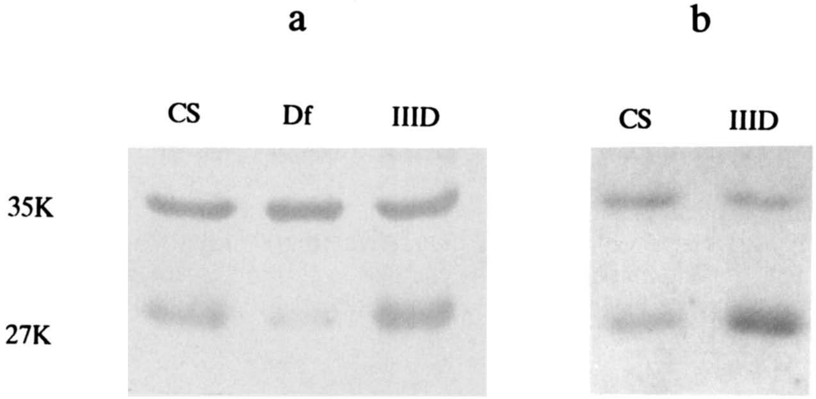

Figure 2. Abundance of the major GST in extracts of malathion-sensitive (CS) and resistant (IIID) adults. Left panel - Western blot, probed with antibody to purified GST from *Drosophila*. Right panel - Coomassie Brilliant Blue- stained gel of SDS-denatured, purified GST. Df (left panel) - extract from flies heterozygous for deficiency of the GST1-1 structural locus. Numbers indicate apparent molecular weight.

indicating that equivalent amounts of total mRNA were present in the two lanes (data not shown).

**Additional proteins with elevated abundance in IIID flies.** Houpt et al. *(10)* showed that selection for malathion resistance resulted in elevated levels of total cytochrome P450 and P450-mediated activities, that was due to an increased level of one particular P450 isoform. They then partially purified P450's from IIID adults, and prepared an antibody to the resulting preparation (Morton, pers. comm.). Given the heterogeneity of the antigen employed, this antibody would be expected to react with multiple proteins, not only P450's. We used that antibody as a probe of Western blots containing total protein from Canton S and IIID adults. As shown in Figure 4, this antibody detects four proteins, of 55,000 da, 47,000 da, 34,000 da, and 18,000 da. that are present in IIID extracts but not in ones from Canton S. The 55,000 da species, a heme-containing protein, is likely the overexpressed P450 (Morton, pers. comm.). Using this antibody as a probe, we screened an expression library of cDNA's from sensitive flies in the vector lambda gt11, and obtained one positive clone. Sequence analysis showed it to be specific for the 34,000 da protein, and based upon sequence similarity to vertebrate GST's, we conclude that this protein is also a GST, designated DmGST2 *(26)*. In contrast to DmGST1, however, no differences in mRNA abundance between control and resistant flies was observed. These data suggest that overexpression of this protein is due to the actions of a different mechanism from that mediating DmGST1-1 expression.

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**Sequence differences in DmGST1 from sensitive and resistant flies.** The above data demonstrate overexpression of DmGST1-1 and DmGST2-1 in flies selected for malathion resistance. It is also possible that selection might result in fixation of alleles with amino acid sequence differences that result in altered function of the protein. To examine this question, we amplified the DmGST1-1 coding sequence, using genomic DNA from strain IIID as a template. The sequence of the entire coding region was then determined. We identified a 6 nucleotide duplication, of the sequence CGTCGA between the second and third nucleotides of codon 25. The effect of this insertion is the addition of two amino acids, aspartic acid and valine, to the IIID protein. Other than these differences, the sequence of the IIID gene is identical to that published by Toung et al. *(23)*.

**Sequence comparisons between GST's from *Drosophila melanogaster* and those of other species.** An alternative means of examining sequence evolution is to compare sequences of both closely and distantly related species. The former comparison may be informative with respect to rates of evolution of amino acid sequence within regions of a particular protein, while the latter resolve questions regarding evolutionary homology among sequences. In particular, having identified a sequence polymorphism

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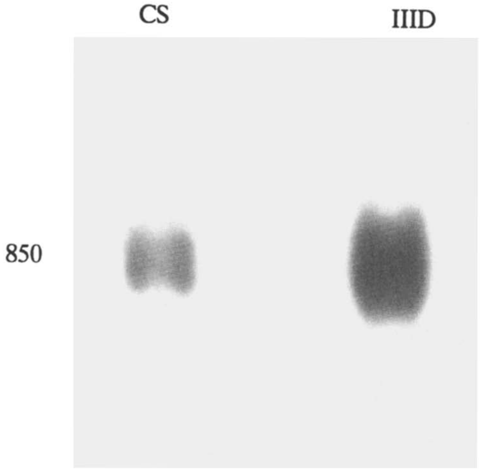

Figure 3. Abundance of mRNA hybridizing to probe for DmGST1-1 in malathion sensitive (CS) and resistant (IIID) flies. Northern blots were prepared as described in Materials and methods, and probed with a clone specific for DmGST1-1 (Morrissey et al., in prep.). Shown is the single hybridizing mRNA species, of 850 bases in length.

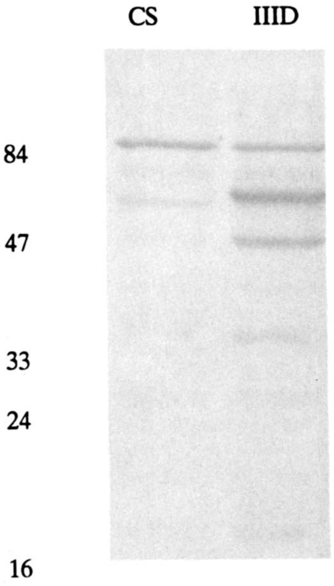

Figure 4. Western blot of proteins from malathion sensitive (CS) and resistant (IIID) flies, probed with antibody to proteins specific to the IIID strain. Numbers indicate molecular weight (in kD) of molecular weight markers.

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associated with resistance, we can ask whether differences in that region of the protein are also associated with species differentiation.

To examine the question of sequence divergence between related species, we determined the nucleotide sequence of the coding region of GST1-1 from *Drosophila simulans*, a sibling species of *Drosophila melanogaster*. Differences between the two sequences are summarized in Figure 5. A total of 21 nucleotide sequence differences were detected, resulting in seven amino acid substitutions. While nucleotide sequence differences were found throughout the protein, amino acid differences were confined to the C-terminal third of the protein (see discussion). No substitutions, silent or missense, were found in the vicinity of the site of the six nucleotide insertion in the IIID allele.

Finally, we compared the sequences of both DmGST1 and DmGST2 to published sequences, in order to determine if sequence similarity between *Drosophila* proteins and those from other organisms, in particular mammals, might be informative with respect to protein function. Toung et al. *(23)* and Wang et al. *(41)* have demonstrated similarity between DmGST1 and its house fly homolog to several other sequence, including one from maize (GST III) and the GST-related bacterial protein dichloromethane dehologenaase *(42)*. We searched for local similarity, using the BLASTP algorithm, that identifies subregions of sequences that show similarity, and determines the level of significance of that similarity. By this approach, two regions of homology were identified, as shown in Figure 6. The first, from spanning a region from amino acid 40 to 80, shows maximum similarity to the sequences described above, and also to the *E. coli* stringent starvation protein *(43)*, a polypeptide not previously associated with GST's. The second region, also shown in Figure 6, spans the regions from amino acids 144-161, and shows homology to four alpha-class GST's *(44)*, two from human and one each from rat and mouse. Similar analysis of DmGST2 revealed the presence of one single region of homology, spanning amino acids 80-140, that has highest similarity to two pi class GST's, one from *Caenorhabditis elegans* and one from mouse (Figure 6b).

**Cross-tolerance of IIID adults to diazinon.** The data described above indicate that selection for resistance to malathion is correlated with elevated levels of two GST's in *Drosophila*, and sequence analysis may provide some insight into the evolutionary forces shaping the structures of these proteins (see discussion). However, while elevated GST's have been reported to be associated with malathion resistance *(45)*, malathion is not known to be a substrate for the enzyme *(46)*. Organophosphates such as parathion and diazinon are, however, metabolized by this enzyme. As cross-resistance to multiple insecticides is a frequent characteristic of resistant lines *(6)*, we asked whether strain IIID might be resistant to diazinon as well as malathion. Dose response curves, measuring lethality, are shown in Figure 7, clearly indicate that these flies are cross-tolerant, with the elevation of

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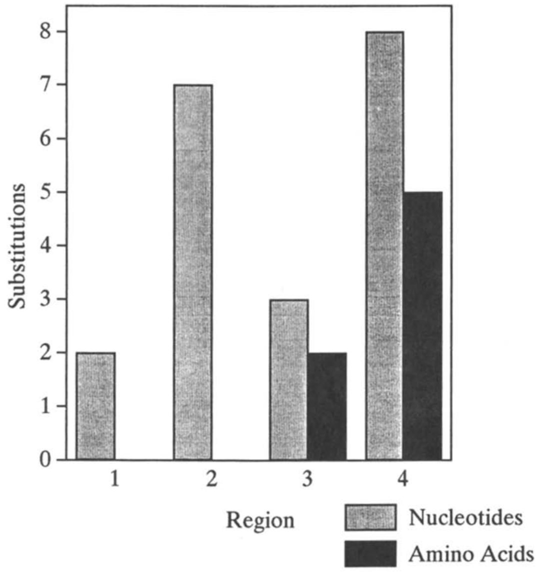

**Figure 5.** Distribution of nucleotide and amino acid differences of Gst1-1 between *Drosophila melanogaster* and *D. simulans*. The 643 base coding region is divided into four regions, with Region 1 being N terminal.

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A.   DmGST1

```
DmGST1        46  KINPQHTIPTLVDNGFALWESRAIQVYLVEKYG 78
Maize IIIA    48  ALNPFGQIPALVDGDEVLFESRAINRYIASKYA 80
DCM           59  KYNPTGQVPILVDGEFTVWESVAIARYVNEKFD 91
SSP           52  DLNPNQSVPTLVDRELTLWESRIIMEYLDERF  84
                    **    * ***      **  *  *

DmGST1       144  GQDYAAGDSLTVADIALV 161
Human Ha     143  GQDYLVGNKLSRADIHLV 160
Rat Yc       143  GQDYLVGNRLSRADVYLV 160
Mouse Ya     143  GQDYLVGNRLTRVDIHLL 163
                  ****  *  *   *  *
```

B.   DmGST2

```
DmGST2        83  LKPTMPMGQMPVLEVDGKRVHQSISMARFLAKTVGLCGATPWEDLQIDIVLDTINDFR 140
Mouse Pi      43  LKPTCLYGQLPKFEDGDLTLYQSNAILRHLGRSLGLYGKNQREAAQMDMVNDGVEDLR 100
C. elegans    41  IKPKMIFGQVPCLLSGDEEIVQSGAIIRHLARLNGLNGSNETETTFIDMFYEGLRDLR  98
                   **    ** *          **    * *    ** *    *    *       * *
```

Figure 6. Regions of sequence similarity between GST's from *D. melanogaster* and other species. Numbers indicate amino acid position (relative to initiation methionine). DCM -dichloromethane dehalogenase *(40)*. SSP - stringent starvation protein *(41)*. Other sequences are from indicated source. Asterisks indicate amino acids identical among all sequences.

In Molecular Mechanisms of Insecticide Resistance; Mullin, C., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1992.

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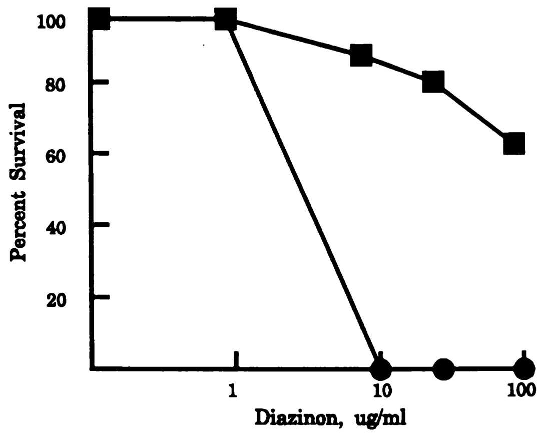

Figure 7. Sensitivity of Canton S (circles) and IIID (squares) flies to malathion. 20 three day-old adults were placed in vials containing the indicated concentrations of diazinon in media consisting of 2% sucrose, 1.5% diazinon. Survivorship was determined after 1 hr. Each point represents the mean of three determinations.

resistance to diazinon, ca. 20-fold, being comparable of that to malathion *(9)*.

## Discussion

The objectives of these studies were to identify gene products that might be associated with evolved pesticide resistance in *Drosophila*, and to determine the nature of the molecular events associated with the acquisition of resistance. Several points emerge from our findings.

**Glutathione S-transferases are overexpressed in malathion-resistant flies.** As was initially reported by Singh and Morton *(9)*, there is elevated GST activity in flies from strain IIID. Such elevation of activity has often been associated with resistance to various insecticides *(47-51)*, and in some cases association with malathion resistance in particular has been reported *(45)*. It is clear that much of this elevation is due to increases in the level of the DmGST1, and that this elevation is paralleled by an increase in mRNA encoding that polypeptide. In addition, we have observed elevated expression of DmGST2-1; compared to DmGST1-1, this is a low abundance protein, and its contribution to total GST activity is uncertain.

Is there a causal relationship between GST expression and malathion resistance? As noted, malathion is not generally thought to be a GST substrate. These flies are cross-resistant to diazinon, a compound whose metabolism by GST's is well established *(46)*. In addition, GST's in many species possess noncatalytic binding activities. The possibility therefore exists that these proteins may function in some transport process that contributes to the metabolism and/or elimination of malathion.

**Overexpression of DmGST1 and DmGST2 is mediated by separate mechanisms.** As noted above, the elevated levels of DmGST1 in strain IIID is paralled by elevated levels of specific mRNA (Figure 3). Preliminary Northern analysis of DmGST2-1 mRNA abundance indicates that such a parallel between mRNA and protein abundance does not exist in this case *(26)*. Thus, in the case of DmGST1-1, differences in transcription levels may underly differences in protein abundance, but such a mechanism cannot account for patterns of abundance of DmGST2-1 observed. Further complicating the picture regarding control is the fact that the GST1-1 gene is on chromosome III, while that for GST2-1 is on chromosome II. It is on chromosome III that the major gene for resistance was mapped in this strain *(10)*.

**Changes in expression of multiple genes is associated with evolved resistance.** We have focused on GST's and their potential involvement with resistance. In addition, this strain exhibits elevated levels of cytochrome P450's *(11)*. Furthermore, as shown in Figure 4, an antibody to proteins elevated in strain IIID detects at least four overexpressed proteins. Two of

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them have been identified - the 55,000 da P450 and the 34,000 da. DmGST2-1. The identity of the remaining two remains to be determined. Finally, Singh and Morton *(9)* reported lowered total esterase activity, likely due to alterations in the structure or expression of acetylcholinesterase. Thus, in this particular case, while a relatively small number of genes may have responded to selection, those genes may have pleiotropic effects on the expression of a large number of structural genes.

**Structural as well as regulatory differences exist between proteins in sensitive and resistant strains.** Sequence analysis of the GST1-1 allele in IIID flies demonstrates the presence of structural as well as regulatory differences associated with resistance. It might be argued that the PCR product we sequenced did in fact result from amplification of a related sequence in the *Drosophila* genome. However, *in situ* and Southern hybridization analysis indicate that under conditions of high stringency, only a single homologous sequence can be detected *(23,24)* We do not know the functional significance of this difference, but it is notable that it occurs near the N-terminus of the protein, within the most highly conserved region. It will be of interest to determine whether it is associated with insecticide resistance in other selected strains.

**Amino acid differences between species are confined to the C-terminal portion of DmGST1.** Comparison of the inferred amino acid sequences of GST1-1 from *D. melanogaster* and *D. simulans* shows that all amino acid substitutions are situated in the C terminal portion of the protein. Recently, the three dimensional structure of a class pi GST from pig was reported; it demonstrates that the protein consists of two domains, the first of which includes amino acids 1-74 and includes most of the glutathione binding domain *(52)*. It is thus not surprising that this region should be conserved. In contrast, the distribution of silent site substitutions is relatively uniform. We are currently determining the sequence of this gene from the remaining members of the melanogaster subgroup of *Drosophila*, in order to better characterize nucleotide and amino acid substitutions associated with species differences.

**DmGST1 contains regions with similarities to different GST families.** The similarity between DmGST1-1 and GST-related sequences from maize and bacteria has been reported previously *(23,41)*. We also detect similarity between a different region this protein and alpha-class GST's from mammals. Similar observations have been made with a GST from *Schistosoma mansoni*, which has regions similar to both class α and class mu mammalian GST's *(53)*. These observations indicate that at least some of the elements found in the major classes of mammalian GST's have a fairly ancient evolutionary origin.

## Conclusion

A number of questions remain open. Our sequence analysis to date has been confined to protein coding regions. Similar analyses of flanking regions would be signficant for two reasons. First, analysis of 5' flanking regions of DmGST1 might allow us to identify regulatory sequences responsible for the different levels of expression observed. Second, by analysis of linked polymorphic sequences or restriction sites, it may be possible to make inferences regarding the evolutionary history of resistance conferring alleles, such as the insertion present in DmGST1 from IIID *(54-57)*.

Our data do not address the question of the frequency of alleles associated with resistance in natural populations. This is a critical question in the context of pest management, in that the rate of response to selection will be dependent on the extent of preexisting variation in field populations. With the advent of PCR, it is now practical to screen large numbers of genotypes for allelic variation. Once alleles have been identified, it may then become possible to develop allele-specific primers that can further streamline the survey process.

The major weakness of the approach used is that it permits us to establish correlations between genes, gene products, and resistance, but it cannot by itself establish causal relationships. It is with respect to this problem, however, that *Drosophila* has potential as a model system, since the availability of facile germ line transformation techniques may allow one to introduce particular genes into the genome and directly determine the effect of gene expression on resistance *(14)*.

Despite much remaining uncertainty, the picture of malathion resistance in *Drosophila* that emerges is one of a complex regulatory system affecting multiple genes, combined with fixation of some structural variants of these genes. The challenge that remains is to further characterize this system in terms of molecular mechanisms and their specific role in ther physiology of pesticide resistance. These objectives are clearly attainable by proper application of genetic, biochemical, and molecular technologies.

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# Chapter 6

# Mechanisms of *kdr* and *super-kdr* Resistance

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Knockdown resistance as exemplified by genetically defined *kdr* strains of the housefly, *Musca domestica*, represents a serious threat to continued use of the pyrethroid insecticides in the field. Pyrethroids are neurotoxins, their major target site being the voltage sensitive $Na^+$-channel of nerve cells. All strains so far investigated by neurophysiological techniques posses a factor which reduces the sensitivity of their $Na^+$-channels to these compounds. This reduction in sensitivity has been correlated with alterations in physicochemical properties of phospholipids and $Na^+$-channel proteins. However, nerve insensitivity associated with axonal conduction can not by itself account for the greater resistance shown by *super-kdr* over *kdr* strains. Since pyrethroids are known to interfere with physiological processes at synaptic junctions, other factors such as perturbation of protein phosphorylation and/ or intracellular $Ca^{2+}$ regulation could well be involved in the *kdr* resistance complex.

The phenomenon of knockdown resistance *(kdr)*, the most common form of resistance against DDT and the pyrethroids *(1,2)*, was first recognised by Busvine *(3)*. The gene for knockdown resistance in the housefly, *kdr*, is a recessive allele found on chromosome 3 *(4-7)*. Resistance is not due to delayed penetration or metabolic factors, but to insensitivity of the nervous system *(8,9)*. This was first shown by Tsukamoto *et al (10)* in the housefly *(Musca domestica)* and subsequently confirmed by several authors in both the larva *(8,11-13)* and adult *(14-18)* of this species.

Although not isolated genetically, *kdr*-like mechanisms have been identified by electrophysiological methods in other Diptera, *Culex quinquefasciatus (12,19)*, *Anopheles stevensi (12)*, *Drosophila melanogaster (20,21)*, in Lepidoptera, *Heliothis virescens (22)* and *Spodoptera littoralis (23)* and in Dictyoptera, *Blatella germanica (24,25)*. No doubt many more examples will be found.

More recently *(9)* a resistance factor has been identified in a Danish strain of the housefly, also on chromosome 3, that conferred an even higher level of resistance to

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pyrethroids *(26)*. This factor, of which there are a number of alleles, was named ***super-kdr***; it was suggested *(26)* that the greater resistance of ***super-kdr*** could result from an enhancement of ***kdr*** nerve insensitivity. Only a few electrophysiological studies have been carried out with ***super-kdr*** houseflies. Some workers *(8,27,28)* did find that the nervous system in this strain was less sensitive to pyrethroids than that of ***kdr*** flies. On the other hand Gibson ***et al*** *(17,18)* found that axons of ***super-kdr*** flies were more not less sensitive to DDT and the pyrethroids than those of ***kdr*** flies.

The voltage sensitive $Na^+$-channel does not appear to be the only site of lesion for pyrethroids and DDT within the nervous system. These compounds also inhibit a number of $Na^+$- and $Ca^{2+}$-dependent ATPases, interfere with phosphorylation of $Na^+$-channel and presynaptic proteins, disrupt $Ca^{2+}$ conductance and intracellular $[Ca^{2+}]$ regulation and interact postsynaptically with $\gamma$ - aminobutyric acid (GABA) and nicotinic acetylcholine (nACh) receptors (see *18,29,30* for references). The present article is concerned with an appraisal of reported sites of lesion of DDT and pyrethroids and their relevance, if any to the ***kdr*** resistance mechanism. We also present new electrophysiological data from houseflies which reinforces the likelihood that differences between ***super-kdr*** and ***kdr*** strains of the housefly can not be accounted for by simple enhancement of the ***kdr*** nerve-insensitivity factor *(18,31)*.

## Mechanisms Associated with *Kdr*

It is clear from the previous section that nerve insensitivity is inexorably linked with ***kdr***. This insensitivity is most likely due to structural alteration of the voltage sensitive $Na^+$-channel *(30)*. An additional factor reported to account for nerve insensitivity is a decrease in numbers of $Na^+$-channels within the nerve membranes *(32,33)*. Changes in physicochemical properties of nerve membrane lipids may also be involved *(34)*. Other factors which may potentially be associated with the ***kdr*** resistance complex include alterations in sensitivity of $K^+$- and $Ca^{2+}$-channels, enzyme systems including $Na^+$- and $Ca^{2+}$-ATPase and second messenger systems involving phosphorylation of proteins associated with $Na^+$-channels and release of synaptic neurotransmitters *(35,36)*. Target sites of pyrethroids and DDT upon the nervous system and the known and suspected sites of modification associated with knockdown resistance are summarised in Figure 1.

## Neurophysiological Studies

A substantial number of neurophysiological investigations have been carried out on insects with ***kdr*** and ***kdr***-like resistance factors (Table I and II). Since these involve studies upon both axonal and synaptic transmission these are dealt with under separate sections.

**Axonal Transmission.** Several effects by pyrethroids upon axonal transmission have been reported; they include induction of hyperexcitability expressed as repetitive activity or bursting, blockage of action potentials, or hyperexcitation followed by block. These effects upon axonal transmission vary from preparation to preparation within the same animal, between species and between compounds. Thus, attempts to designate pyrethroids into two types *(37)*, based on their abilities to cause repetitive firing in axons (Type I; without the $\alpha$ - cyano group), or depolarise nerve axons and terminals (Type II; with the $\alpha$ - cyano group: *39,84)* are not always straightforward *(17,57)*. Type I are generally better knockdown agents than Type II compounds *(12)*. This is attributed

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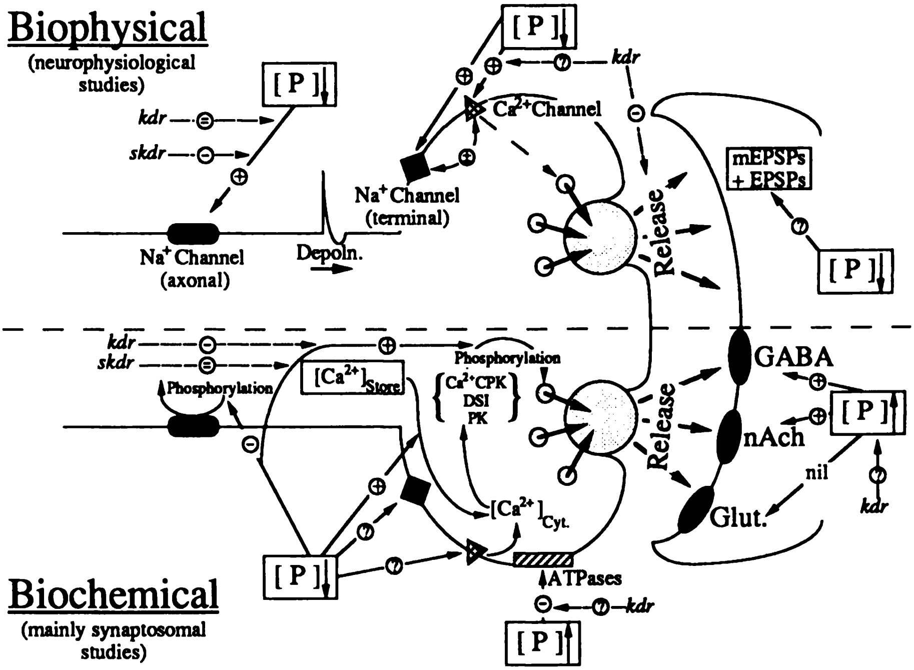
Biophysical
(neurophysiological studies)
[P]↓
kdr ⊖
skdr ⊖
⊕
Na⁺Channel (axonal)
Depoln.
Na⁺Channel (terminal)
[P]↓
⊕
⊕
kdr
Ca²⁺Channel
⊖
⊕
Release
mEPSPs + EPSPs
[P]↓
kdr
skdr
⊕
Phosphorylation
[Ca²⁺]Store
Ca²⁺CPK
DSI
PK
⊖
⊕
[Ca²⁺]Cyt.
Release
GABA
⊕
[P]↑
⊕
nAch
nil
Glut.
kdr
[P]↓
ATPases
⊖
kdr
[P]↑
Biochemical
(mainly synaptosomal studies)

Figure 1. Target sites of pyrethroids (P) on the nervous system and the known and suspected sites of modification associated with knockdown resistance: An upward or downward directed arrow accompanying the "P" symbol indicates high ($>10^{-6}$ M) or low ($<10^{-6}$ M) concentrations of pyrethroid, respectively; "+", indicates a site specific excitation or enhancement of activity; "-", indicates a site specific inhibition or reduction in activity; Glut., glutamate receptor; $Ca^{2+}$CPK, calcium-dependent calmodulin stimulated protein kinases; DSI, dephosphosynapsin I; PK, protein kinases. Note *kdr* and *super-kdr* factors effect i) axonal $Na^+$-channel sensitivity; ii) $Ca^{2+}$ -dependent phosphorylation of proteins involved in transmitter release.

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Table I. Electrophysiological Investigations on *kdr* Resistance in *Musca domestica*

| *Genetic Factor* | *Application/ Recording Site* | *Effect Recorded* | *Resistance Factor (R/F)* | *Author* |
|---|---|---|---|---|
| *kdr* (A) | Thoracic Ganglia/ Flight Muscle | Latency to Muscle Potential "Uncoupling" | $10^4$ c,d,e | Miller *et al* *(15)* |
| *kdr* (L) | Bath/ Muscle and Sensory Axons | Change in Evoked EPSP profile/ Elevated Spike Discharge | $1/10^3$ a | Osborne and Hart *(11)* |
| "<br>*Super-kdr* (L) | Bath/ Muscle and Sensory Axons | Change in Evoked EPSP profile/ Elevated Spike Discharge | $1/10^3$ a<br>$1/10^4$ a | Nicholson *et al* *(8)* |
| *kdr* (L) | Bath/ Muscle | Elevated mEPSP Frequency | $10^2$ h | Miller *et al* *(99)* |
| " | Bath/ Motor and Sensory Axons | Elevated Spike Discharge | $10^5$ a, (b,p) | Osborne and Smallcombe *(48)* |
| " | Bath/ Muscle | Elevated Evoked mEPSP Frequency | 33 a,(i,d,j) | Salgado *et al* *(12)* |
| " | " | " | - o,p | Salgado *et al* *(13)* |
| *kdr* (A)<br>*Super-kdr* (A) | Thorax/ Flight Muscles | Latency to Evoked "Burst Discharge"/ Threshold Dose | 1/8 a<br>1/32 a | Scott and Georghiou *(27)* |
| *kdr* (A)<br>*Super-kdr* (A) | Thoracic Ganglia/ Femur Muscle | Latency to Elevated Muscle Discharge | $10^2$ k<br>$10^3$ k,(b) | Ahn *et al* *(28)* |
| *kdr* (A)<br>*Super-kdr* (A) | Bath/ Peripheral Axons | Threshold Dose to Elevate Spike Discharge | $10^6$-$10^7$ b,i<br>$10^5$-$10^6$ b,i | Gibson *et al* *(17)* |
| *kdr* (A)<br>*Super-kdr* (A) | Pretarsus/ Mesothoracic Femoral Nerve | Threshold Dose to Elevate Spike Discharge | $10^2$-$10^6$ b,i,m,n<br>$10^2$-$10^6$ b,i,m,n | Gibson *et al* *(18)* |

(L) Larvae; (A) Adult. Pesticide (s) assayed:- $^a$, Permethrin; $^b$, DDT; $^c$, Tetramethrin; $^d$, RU11679; $^e$, Cismethrin; $^h$, Decamethrin; $^i$, Deltamethrin; $^j$, Fenvalerate; $^k$, Resmethrin; $^m$, RU15525; $^n$, BTG 502; $^o$, TEA; $^p$, Aconitine; ( ), Pesticide (s) not used to calculate R/F value shown.

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**Table II. Electrophysiological Investigations on *kdr* - like Resistance**

| *Species* | *Application/ Recording Site* | *Effect Recorded* | *Resistance Factor (R/F)* | *Author* |
|---|---|---|---|---|
| *Musca domestica* (A) $^{q}$ | Thoracic Ganglia/ Femur Muscle | Elevated Spike Discharge | $>1^{b}$ | Tsukamoto *et al* *(10)* |
| *Spodoptera littoralis*(L) | Bath/ Excised Abdominal Nerve cord | Latency to Nerve Excitation/ Block | 2/ 1 $^{a, (f)}$ | Gammon *(23)* |
| *Anopheles stephensi* (L) | Bath/ Muscle | Elevated mEPSP Frequency | $10^{2}$ $^{a}$ | Omer *et al* *(19)* |
| *Musca domestica* (A) $^{q}$ | Thoracic Ganglia/ Flight Muscle | Latency to Muscle Potential "Uncoupling" | $>43$ $^{c, (a,g)}$ | DeVries and Matsumura *(98)* |
| *Blattella germanica* (A) | Bath/ Ventral Nerve Cord | Latency to Elevated Spike Discharge | 3 $^{a, (b)}$ | Scott and Matsumura *(24)* |
| *Culex quinquefasciatus* (L) | Bath/ Muscle | Elevated mEPSP Frequency | $10^{4}$ $^{a}$ | Miller *et al* *(99)* |
| *Anopheles stephensi* (L) | " | " | $10^{2}$ $^{a}$ | " |
| *Culex quinquefasciatus* (L) | " | Elevated Evoked mEPSP Frequency | $10^{3}$ $^{a}$ | Salgado *et al* *(12)* |
| *Heliothis virescens* (L) | " | Latency to Elevated mEPSP Frequency | 10-50 $^{a,(j)}$ | Nicholson and Miller *(22)* |
| *Blattella germanica* (A) | Bath/ Ventral Nerve Cord | Latency to Block of Spike Discharge | 2-5 $^{a,c,(b,p)}$ | Umeda *et al* *(25)* |
| *Drosophila melanogaster* (A) | Abdomen/ Flight Muscles | Latency to Evoked "Burst Discharge" and Spontaneous Activity | 2 $^{l}$ | Bloomquist *et al* *(20)* |
| *Drosophila melanogaster* (A) | Topical/ Anterior Thoracic Connectives | Latency to "Burst Discharge" and Spontaneous Activity | $>1^{i}$ | Peyronnet *et al* *(21)* |

(L) Larvae; (A) Adult. Pesticide (s) assayed:- $^{a}$, Permethrin; $^{b}$, DDT; $^{c}$, Tetramethrin; $^{d}$, RU11679; $^{e}$, Cismethrin; $^{f}$, Cypermethrin; $^{g}$, Ethanoresmethrin; $^{i}$, Deltamethrin; $^{j}$, Fenvalerate; $^{l}$, Fenfluthrin; $^{()}$, Pesticide (s) not used to calculate R/F value shown; $^{q}$, Not genetically defined as *kdr*.

to their ability to induce repetitive firing, but the latter is poorly correlated with toxicity *(13,38,39)*.

These neurophysiological effects have all been related to pyrethroids perturbing gating kinetics of the voltage dependent $Na^+$-channel, in particular by slowing down inactivation of the channel, thus prolonging the $Na^+$-current during membrane excitation *(40)*. However, both activation and inactivation processes can be retarded *(41)*.

The actual site of binding of pyrethroids to the $Na^+$-channel has been the subject of intense interest. Pharmacological evidence (Figure 2) indicates that there are 5 distinct binding sites for neurotoxins *(42)*. Work with DDT and the pyrethroids has shown that these neurotoxins do not bind to these five sites and therefore an additional binding site (site 6; Figure 2) has been proposed *(43, 100)* (It is noted here that other authors e.g. Bidard *et al* *(101)*, have designated the pyrethroid binding site as site 5). However, site 6 is allosterically coupled to site 2 which binds a range of other lipophilic neurotoxins such as batrachotoxin, veratridine, aconitine, grayanotoxin and a more recently recognised class of insecticide, the *N*- alkyl amides *(43-47)*.

Studies on *kdr* strains have indicated that they show no resistance to site 1 or site 3 toxins, since they exhibit no insensitivity to tetrodotoxin or scorpion *(Leiurus)* venom respectively, either from neurophysiological *(12,48)* or bioassay data (*16,49*). On the other hand *kdr* or *kdr*-like strains are resistant to some lipophilic site 2 toxins, namely veratridine (veratrin), aconitine and grayanotoxin I *(12,48,102)* but not to others, such as the N-alkyl amides *(44)* and batrachotoxin *(50)*. Extension of *kdr* resistance from site 6 to site 2 toxins indicates a close topographical and/or stereological relationship between them. Indeed, even for batrachotoxin there is allosteric coupling between the binding of this compound and pyrethroids, and moreover, this coupling is altered in *super-kdr* flies (50). Nevertheless, the failure of *kdr* to protect against all site 2 toxins does pose a dilemma. An explanation may be that site 2 comprises several interelated but distinct binding sites. Further detailed pharmacological investigations of this site are clearly required to settle this question.

At the present state of knowledge it is not possible to link pharmacological data with specific sites on the $Na^+$-channel molecule *(51)*. The primary structure of the $\alpha$-subunit of this channel, is a glycoprotein, which has been sequenced for vertebrates (rat; *52*); and insects *(Drosophila; 53)* and is shown to be remarkably conserved, but it has not yet been determined for the housefly. When it is known, together with the changes in protein structure associated with the different *kdr* strains, it should be possible not only to identify the precise binding sites for pyrethroids, but to gain a greater understanding of the functions of the respective components of the $Na^+$-channel. Nevertheless some progress with the later has been made; an intracellular peptide segment of the channel has been identified, using antibody binding techniques, which is associated with $Na^+$-channel inactivation *(54)*.

Pyrethroids are not exclusively $Na^+$-channel toxins; they also act upon $K^+$- *(55)* and $Ca^{2+}$-channels *(56-58)*. However, these effects are not considered to be significant in the lethality of pyrethroids *(29,40)*. $K^+$-channel effects, for instance are obtained only at concentrations well above those required to perturb $Na^+$-channels. Sattelle and Yamamoto *(29)* considered that results with insect neurosecretory cells in relation to effects of pyrethroids upon $Ca^{2+}$-channels *(56)* were inconclusive because a small amount of $Na^+$ was required in the extracellular fluid to support axonal conduction and it could not be ruled out that a small proportion of the voltage-dependent channels were $Na^+$-dependent, thus accounting for their susceptibility to pyrethroids. This may be true for neurohaemal nerve axons of central origin, but cannot be so for the peripheral link

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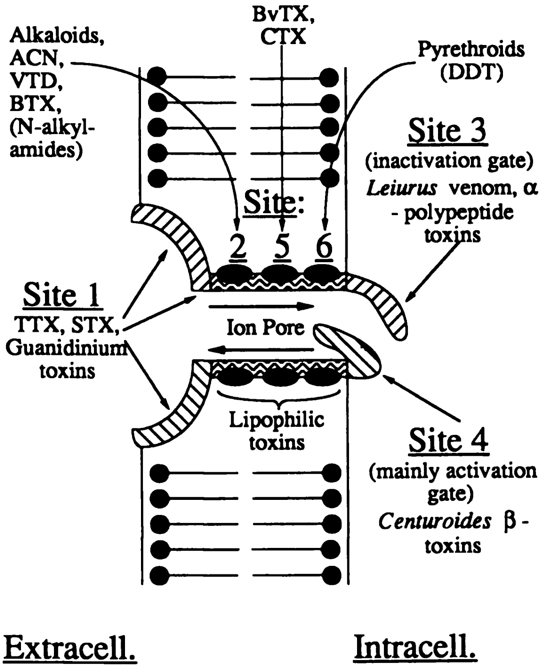
BvTX, CTX
Alkaloids, ACN, VTD, BTX, (N-alkyl-amides)
Pyrethroids (DDT)
Site 3
(inactivation gate)
*Leiurus* venom, α - polypeptide toxins
Site:
2 5 6
Site 1
TTX, STX, Guanidinium toxins
Ion Pore
Lipophilic toxins
Site 4
(mainly activation gate)
*Centuroides* β - toxins
Extracell.
Intracell.

Figure 2. A diagram of the axonal sodium channel indicating six known pharmacologically isolated toxin binding sites (based on data from refs. *42,100*): The straight line filled regions represent hydrophilic domains, whereas the wavy line filled regions indicate lipophilic domains of the channel; The geographical order of the intramembrane sites 2,5 and 6 are displayed arbitrarily; ACN, aconitine; VTD, veratridine; BTX, batrachotoxin; CTX, ciguatoxin; TTX, tetrodotoxin; STX, saxitoxin; BvTX, brevetoxin.

nerve neurons, which are also susceptible to pyrethroid action, because the inward current for their voltage sensitive channels is carried exclusively by $Ca^{2+}$. Moreover there is little doubt that the evidence for pyrethroid action upon $Ca^{2+}$-channels is strengthening *(59,60)*, particularly at synaptic contacts (see next section).

Many of the neurophysiological studies upon *kdr* and *kdr*-like strains have utilised preparations which included synaptic elements (Table I and II) this makes it difficult to differentiate insensitivity factors concerned only with axonal conduction from those concerned exclusively with synaptic transmission (see section on Presynaptic Effects). Studies on peripheral axonal preparations isolated from the CNS are relatively few *(8, 11,17,18,48)*, but in non of these were there any synaptic component. They were concerned with determining the threshold concentrations of pyrethroids required to induce repetitive firing in silent preparations or elevate the spontaneously occurring firing rate in peripheral nerves. All clearly showed that axons from *kdr* strains of both adult and larval forms of the housefly are at least 2 to 3 orders of magnitude less sensitive to DDT and the pyrethroids than the susceptible (Cooper) strain (Table I). In an early study *(8)* on sensory nerves of housefly larvae it was shown that ***super-kdr*** axons were 10 times less sensitive to pyrethroids than *kdr* axons; this was not confirmed by more recent studies *(17,18)* on sensory axons of adult ***super-kdr*** flies, where if anything (Figure 3), the nerve insensitivity factor was *less* than in *kdr* flies *(17,18)*. Gibson *et al (18)* also investigated the effects of an *N*- alkyl amide, BTG 502, upon *kdr* and ***super-kdr*** strains of the housefly, since flies possessing these factors have shown marked negatively correlated cross-resistance to *N*- alkyl amides, that is they are more susceptible to them (toxicity rank order of susceptibility: ***super-kdr>kdr>***Cooper) than the reference Cooper strain *(44)*. It was found that whilst nerves of *kdr* flies had a reduced nerve insensitivity to BTG 502, some 100 fold compared with Cooper flies, nerves from ***super-kdr*** flies did not (Figure 4). Thus axonal insensitivity factors in adult flies by themselves, apparently do not account for the relative susceptibilities of *kdr*-resistant strains of housefly to pyrethroids or *N*- alkyl amides obtained from toxicity data *(18)*. However, this still leaves unexplained, the dilemma that in the larval form, ***super-kdr*** nerves were less sensitive than those of *kdr*. Since the observations on the larvae were of a preliminary nature, further studies on the larval nerves might resolve this disparity. In addition toxicological data are only known for the adult fly.

**Synaptic Transmission.** Pyrethroids have been reported to perturb both presynaptic and postsynaptic events *(29,40,61)*.

**Presynaptic Effects.** Pyrethroids have been shown to exert physiological lesions at synaptic contacts in both insects and vertebrates *(12,13,62,63)*.

Salgado *et al (12,13)* have clearly shown that pyrethroids exert a presynaptic effect by depolarising nerve terminals that innervate body-wall muscles of housefly larvae. Such depolarisations are considered to be induced via actions of pyrethroids upon voltage-dependent $Na^+$-channels at the axon terminals *(12,13)*. Depolarisation of the nerve terminals has three effects. First it can cause block of axonal conduction in the nerve terminals (early block). Second it elevates the rate of release of miniature excitatory postsynaptic potentials (mEPSPs) inducing depletion of vesicles and hence neurotransmitter from the nerve terminals *(64)* thus eventually causing block (late block) of synaptic transmission. Third it can induce repetitive firing (backfiring) in the nerve terminals in both insect *(14,65)* and vertebrate *(66-68)* neuromuscular junctions. Repetitive firing of nerve terminals was negatively correlated with toxicity, whilst it did

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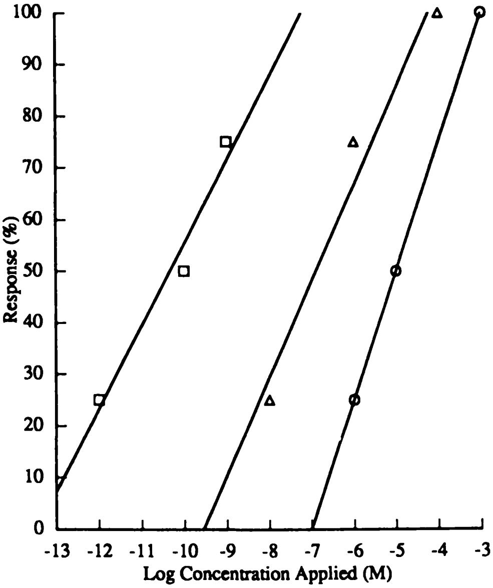

Figure 3. Dose-response lines for RU 15525 calculated from 'increase in firing rate' data for: Cooper, squares; *kdr*, circles; *super-kdr*, triangles (Adapted from ref. *18*). Note *super-kdr* flies are more susceptible than *kdr* flies in this assay.

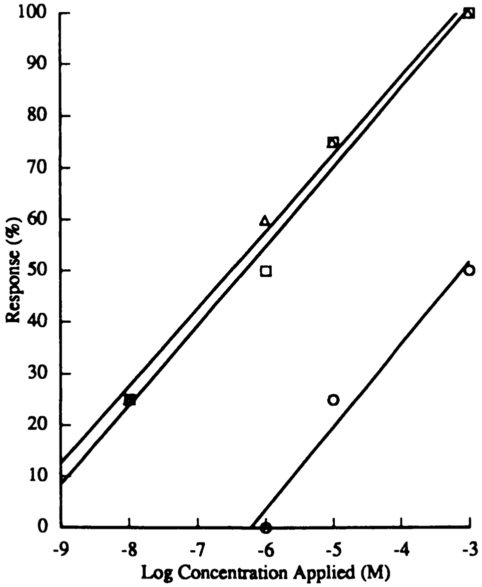

Figure 4. Dose-response lines for the lipid amide, BTG 502, calculated from 'increase in firing rate' data for: Cooper, squares; *kdr*, circles; *super-kdr*, triangles. Note virtual superimposition of Cooper and *super-kdr* strains (Adapted from ref. *18*).

correlate with the knockdown ability of pyrethroids; toxicity was found to correlate better with the ability of the compound to raise mEPSP frequency *(12,13)*.

Dipteran larvae with *kdr* or *kdr*-like resistance *(12,13,19,22)* have presynaptic neuromuscular terminals that are less sensitive to pyrethroids and DDT. Compared with susceptible strains it takes longer for a given concentration of pyrethroid to induce an elevation in rate of firing of mEPSPs or repetitive activity in the motor nerves and it requires a higher concentration to produce an elevation in mEPSP frequency (Table I and II).

There are a number of studies where recordings have been made from flight muscles *(15,20,27)*, from central nervous connectives *(21,23-25)* or from the femur *(10,28)* of *kdr* or *kdr*-like resistant insects. In all these cases insensitivity of the nervous system was found (Table I and II), but since some form of synaptic element was involved, these results cannot determine whether resistance sites were associated exclusively with axonal conduction or synaptic transmission or with both. Where *kdr* and *super-kdr* strains have been compared in these types of preparations differences in sensitivity have been found. *Super-kdr* flies *(27)* were 4 times less sensitive to pyrethroids than *kdr* flies; Ahn *et al (28)* found a tenfold difference between these two strains (Table I).

**Postsynaptic Effects.** Evidence is accumulating which suggests that pyrethroids interfere with ligand-gated ion channels of postsynaptic membranes (*29,61*). Effects upon GABA mediated synaptic contacts have been reported for type II, but not for type I pyrethroids particularly in vertebrate preparations where they perturb chloride fluxes *(40,69)*. Their actions upon insect and crustacean synapses *(39,70,71)* have been challenged *(29,72)* on the grounds that much higher concentrations, some several orders of magnitude, of type II, α - cyano pyrethroids are required to produce effects compared with those upon voltage-gated $Na^+$-channels. Nevertheless, Ramadan *et al (73)* have argued that whilst there is a poor correlation between potencies of pyrethroids and their toxicities, the stereospecificity of pyrethroid action at GABA receptors could well contribute to the toxicity of some type II compounds.

Reports that pyrethroids disrupt nicotinic, cholinergic transmission *(66,68)* are somewhat controversial *(74)*. More recent work *(62)* further suggests that pyrethroids are active at nicotinic synapses. Deltamethrin suppressed acetylcholine-induced currents in identified cockroach central motor neurons *(29)* and snail neurons *(75)*. However, even here relatively large concentrations (in the μM range) were needed to achieve effects on postsynaptic receptors.

Seabrook *et al (76)* found that, the type II compound, cypermethrin, had no significant postsynaptic effect at neuromuscular junctions in housefly larvae where presumably the putative neurotransmitter is glutamate. They concluded that all actions of pyrethroids could be explained from presynaptic effects.

Irrespective of postsynaptic receptors being legitimate target sites for pyrethroids, alterations of chemically-gated ligands have not yet been reported in association with *kdr* resistance. If GABA or cholinergic receptor actions do contribute significantly to the *kdr* resistance against type II pyrethroids, it would not explain why *kdr* factors are so effective against type I pyrethroids *(18)*.

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## Biochemical Studies

A number of effects of pyrethroids and DDT upon enzymes including various ATPases have been reported *(29,61)*. Recent evidence implicates pyrethroids with second messenger systems particularly those involved with phosphorylation of both the voltage-dependent $Na^+$-channel and protein molecules concerned with neurotransmitter release *(18)*. Differences in the physicochemical composition of membrane lipids *(35)* and in the numbers of $Na^+$-channels *(32)* between resistant and susceptible strains have also been implicated in *kdr* resistance mechanisms.

**Effects upon ATPases.** Certain ATPases including the cell membrane associated $Na^+$-, $K^+$-ATPase, mitochondrial $Mg^{2+}$-ATPase, and $Ca^{2+}$-ATPase have been shown to be inhibited by DDT and pyrethroids. Inhibition of these enzymes, with the possible exception of the plasma membrane-associated $Ca^{2+}$-*ecto*-ATPase *(77)*, occurs only at concentrations of pyrethroids well above those needed to effect the $Na^+$-channel (e.g. about $10^{-5}$ M for $Na^+$-, $K^+$-ATPase and $Mg^{2+}$-ATPase from cockroach nerve cord; *78*) and could well be non-specific *(79)* and, hence, unlikely to be significant in the lethality of these compounds *(29)*. Inhibition of $Na^+$-, $K^+$-ATPase in axons by ouabain, only produces effects upon axonal conduction in the long term and does not mimic symptoms of pyrethroid poisoning *(80)*. However, sensory nerves are sensitive to ouabain and dinitrophenol; both of these compounds induce increases in rates of afferent discharge *(81)*. On the other hand, inhibitors of oligomycin-sensitive mitochondrial ATPase had no effect upon axonal conduction or sensory discharge in crustacean muscle receptor organs *(82)*.

$Ca^{2+}$-*ecto*-ATPase, is an enzyme considered to reside in the exterior regions of the axolemma, and is thought to be of importance in maintaining a high level of $Ca^{2+}$ at the outer surface of the cell membrane *(83)*. This enzyme has been shown to be inhibited by concentrations of DDT similar to those which perturb axonal conduction *(77)*. Such inhibition would be expected to lower the concentration of $Ca^{2+}$ in the outer region of the axolemma thereby leading to instability and hyperexcitability of the axon. Indeed, Ghiasuddin *et al (35)* have reported a reduced sensitivity of $Ca^{2+}$-ATPase in brain homogenates from DDT-resistant strains of the German cockroach. It was shown later that *kdr*-like resistance factors were involved, extending cross-resistance to pyrethroids *(84)*. More recent work on this $Ca^{2+}$-ATPase has suggested that it is likely to be a protein kinase-phosphatase which is sensitive to free $[Ca^{2+}]$. Chang and Plapp *(32)* have also shown that the binding of DDT and pyrethroids to the receptor site in the nerve membrane is affected by $[Ca^{2+}]$. This prompted Rashatwar *et al (36)* to study the effects of $Ca^{2+}$ upon $Na^+$ transport in synaptosomes of housefly brain in susceptible, *kdr* and *super-kdr* strains. They found that the stimulatory effect of $Ca^{2+}$ upon phosphorylation by $Ca^{2+}$-ATPase (i.e., $Na^+$-, $Ca^{2+}$-protein kinase-phosphatase) was less in both resistant strains, but was even less in *super-kdr* than in *kdr* preparations. They also found that $Ca^{2+}$- stimulated uptake of $Na^+$ into synaptosomes was lower in resistant strains. It was therefore concluded that there are intrinsic differences in $Na^+$- and $Ca^{2+}$-transporting systems between susceptible and resistant strains of fly, but the question remained as to whether these differences could be solely attributed to differences in the $Na^+$-channel.

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**Effects upon Phosphorylation Mechanisms.** It is becoming increasingly clear that second messenger processes such as cAMP-dependent and $Ca^{2+}$/phosholipid-dependent phosphorylation mechanisms play a fundamental role in the functioning of ion channels in that they regulate membrane excitability *(85,86)*. For example phosphorylation of the α - subunit of the rat brain $Na^+$-channel has been shown to reduce inward current flow *(87)*. At synapses, depolarization is accompanied by phosphorylation of a number of proteins induced by $Ca^{2+}$-activation of cAMP-, calmodulin-, and phospholipid -stimulated protein kinases *(88,89)*.

Pyrethroids are able to affect phosphorylation reactions in axonal and synaptosomal preparations from both invertebrates and vertebrates *(58,90-94)*. Deltamethrin at $10^{-11}$ to $10^{-7}$M in lobster axons inhibited $Ca^{2+}$-calmodulin-dependent protein kinases and inhibited $Ca^{2+}$-sensitive phosphorylation of a 260 kDa. protein that probably comprised the $Na^+$-channel *(93)*. In squid synaptosomes deltamethrin, at concentrations as low as $10^{-13}$M, induced a prolonged elevation in phosphorylation of a number of key synaptic proteins including $Ca^{2+}$-calmodulin-dependent protein kinase and synapsin I *(92)*. Both phosphorylation of dephosphosynapsin I and activation of $Ca^{2+}$-calmodulin-dependent protein kinase facilitate transmitter release *(92)*. Enan and Matsumura *(94)* found that neither $Na^+$- or $Ca^{2+}$-channel blocking agents or removal of external $Ca^{2+}$ could completely abolish phosphorylation induced by deltamethrin in rat brain synaptosomes. Deltamethrin was also able to stimulate the release of $Ca^{2+}$ from intracellular stores by inducing break down of phosphoinositides to inositol phosphates and diacylglycerols. Inositol triphosphate itself, has been shown to elevate cytosolic $[Ca^{2+}]$ from intracellular stores *(95)*. Since all these effects are produced at concentrations similar to those which perturb the biophysics of voltage-sensitive $Na^+$-channels they could well be of significance in the toxic actions of pyrethroids.

Several workers have reported that type II pyrethroids are more effective at inducing phosphorylation of proteins and have different phosphorylation activities than type I pyrethroids *(90,91,95)*. This could relate to the differential effects between type I and II pyrethroids noted by Salgado *et al (12,13)* upon presynaptic terminals in neuromuscular synapses of Dipteran larvae. Type II compounds elicited increases in rate of mEPSP frequency at much lower concentration than type I compounds. Not only that but a lowered sensitivity to pyrethroids of intraterminal $Ca^{2+}$-regulatory mechanisms and associated phosphorylation processes could also contribute towards the resistance mechanism of *kdr* strains. As we have seen progress in this field is beginning to emerge (see previous section; *36*).

**Differences in Membrane Lipids.** Chialiang and Devonshire *(34)* found that the membrane lipids from Cooper, *kdr*, *super-kdr* strains of the housefly had different transition temperatures, of 14, 19 and 21°C respectively. It is not readily apparent how these differences might relate to $Na^+$-channel insensitivity *(30)*, but it possible that these differences modify the solubility of DDT and pyrethroids in the lipid bilayer of excitable membranes or impede their access to the $Na^+$-channel *(18,48)*. In this context DDT is known to alter membrane fluidity, particularly in cholesterol-enriched membranes such as are found in rat brain microsomes *(96)*. This action might distort the protein-lipid interfaces of $Na^+$-channels which may possibly effect their gating kinetics.

**Differences in Numbers of $Na^+$-Channels.** It has been suggested that *kdr* resistance might be accounted for on the basis of a reduced number of $Na^+$-channels in nerve membranes *(32,33)*. For example, Rossignol *(33)* reported a 40-60% reduction in

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the number of channels in membranes of *kdr* flies. It has also been shown in a *no action potential temperature-sensitive* mutant of *Drosophila (97)* that a reduced $Na^+$-channel number apparently does confer resistance to pyrethroids. However, other workers were not able to repeat or were not convinced of these observations for *kdr* strains of houseflies *(30,50,51)*.

## Neurophysiological Experiments on Whole Houseflies

In a recent attempt to obtain a better correlation between perturbations of nervous activity and resistance factors, we have investigated the effects of pyrethroids upon the electrical activity originating in the neck connectives of intact flies (strains: Cooper-susceptible wild phenotype; 538ge-resistance factor $kdr_{Latina}$; 530-resistance factor $super\text{-}kdr_{3D}$). This preparation includes synaptic inputs from central ganglia and the peripheral nervous system and therefore reflects pyrethroid interactions with all nervous elements.

For recording purposes, a stainless steel pin electrode was inserted into the neck connectives and extracellular electrical activity was recorded relative to a second earthed pin which was inserted into the abdomen. All experiments were carried out at 23.4 ± 1.5°C which lay above the lipid transition temperatures of all three strains of fly *(34)*.

Action potentials from the neck connectives were recorded for periods of 10 minutes before, and 50 minutes after, application of the insecticide, kadethrin. Doses were administered in the range 16.8pg-16.8 μg directly to the thorax in 0.5μl methylethylketone using half segments of 1μl Microcaps. Action potentials were collected and counted by on-line data logging techniques as previously published *(17,18)*. Data were further processed with an Olivetti PCS 286 computer and expressed graphically (Figure 5a,b) using the Jandel Scientific Sigmaplot graphics package.

Although the three strains are clearly different in their electrophysiological response to kadethrin (Figure 5a,b) there are some similarities in their dose-dependent profiles. In agreement with previous work *(18)* at lower doses of kadethrin (< Cooper $LD_{50}$ = 150ng) there is a progressive increase in firing rate with increase in dose, such that the rank order of susceptibility to elevation of the firing rate is Cooper >>*super-kdr* ≥ *kdr*. At these lower doses, *kdr* flies paralleled *super-kdr* flies in their response, but at higher doses (>Cooper $LD_{50}$), the maximum increase in firing rate obtained in *kdr* flies rose to a much greater level (600% *kdr*; 250% and 300% respectively for Cooper and *super-kdr*) compared with the other two strains (Figure 5a,b).

In addition, a depression in the firing rate occurred in all three strains at higher doses. In Cooper the down turn in the dose-response curve occurred at approximately 20ng, in *kdr* at 200ng and in *super-kdr* 2000ng, i.e. the susceptibility of the three strains to a depression in the firing rate is Cooper >*kdr* >*super-kdr*. These results correlate well with the rank order obtained from toxicity data and to some extent substantiates the relationship between block of neural activity and resistance factors reported earlier *(17)*.

From these data it appears that the point of down turn in the dose-response curve is a better indicator of levels of resistance than the threshold levels required to elevate nervous activity. Therefore, determining threshold and $EC_{50}$ values of pesticide levels needed to elevate the firing rate of axons, which have formed the basis of many previous studies may not be a reliable guide for determining resistance levels by electrophysiological means.

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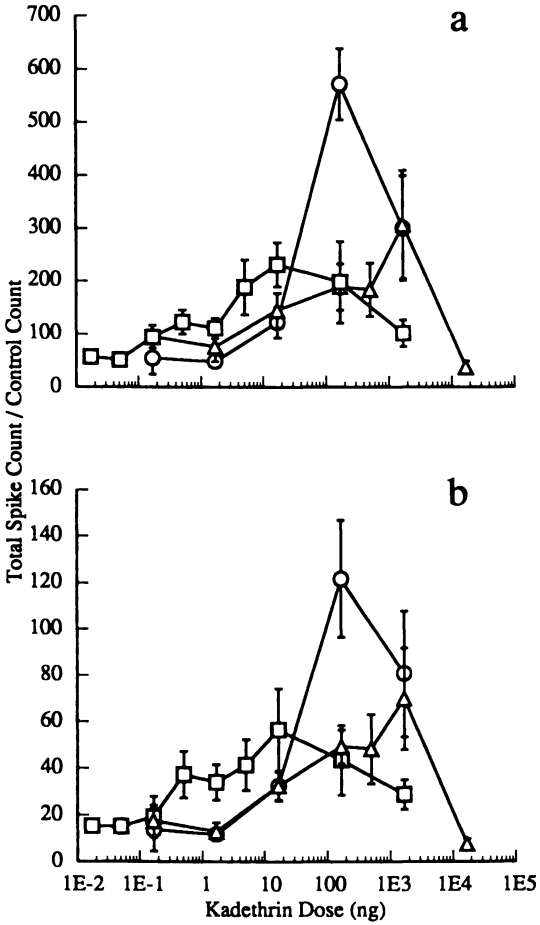

Figure 5. The relative total electrical activity of the neck connectives for: Cooper, squares; *kdr*, circles; *super-kdr*, triangles; in response to doses of kadethrin, a; over the 50 minute test period, b; over the initial 10 minute test period. Points are shown ± sem (n=6) (5a, adapted from ref. 31).

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## Conclusions

All strains of housefly with *kdr* and insects with *kdr*-like resistance factors have been shown by electrophysiological investigations to possess a nerve insensitivity factor to DDT and the pyrethroids. This factor confers resistance to some site 2 lipophilic neurotoxins (e.g. aconitine, veratridine) but not to those of hydrophilic sites 1 and 3. It is further apparent, both from studies with pyrethroids and N-alkyl amides, that the degree of nerve insensitivity differs between *kdr* and *super-kdr* strains indicating that their $Na^+$-channels are different. Our observation that nerves of *kdr* can support a higher rate of firing than *super-kdr* flies also concurs with this view. Perhaps the most interesting finding is that the $Na^+$-channel insensitivity factor in axons of adult *super-kdr* flies is, if anything, less well developed than that in *kdr* strains. Thus axonal insensitivity is insufficient by itself to account for the higher resistance shown by *super-kdr* over *kdr* flies.

Since the *kdr* complex is not associated with factors such as delayed penetration or metabolic degradation of pesticides, it is likely that some other site-insensitive mechanism(s) apart from that of the $Na^+$-channel exists in the insect nervous system. Neurophysiological data from preparations which include synaptic elements show that *super-kdr* flies are less sensitive to pyrethroids than *kdr* flies and that such insensitivity is a property of the presynaptic rather than the postsynaptic region. Biochemical evidence indicates that pyrethroids alter the rate of phosphorylation of a number of key presynaptic proteins, particularly those involved with transmitter release. Many of these phosphorylation reactions are $Ca^{2+}$-dependent. In *kdr* and even more so in *super-kdr* strains these phosphorylation processes are less sensitive to $[Ca^{2+}]$.

The *kdr* resistance factor thus appears to be a complex one which at our present state of knowledge involves at least two site-insensitive mechanisms, one of which is associated with the $Na^+$-channel and the other with presynaptic events possibly involving calcium regulation and associated phosphorylation of neuronal proteins. These could act synergistically to produce the differing degrees of resistance seen between *kdr* strains. Other reported changes such as a reduction in number of $Na^+$-channels and changes in transition temperature of membrane lipids may be additional or complimentary factors. Differences in sensitivity of both ligand- and other voltage-gated ion channels need closer investigation regarding their contribution to *kdr* resistance.

Our major conclusions strongly suggest that the voltage sensitive $Na^+$-channel is not the sole site of action of the pyrethroid insecticides. This view at present flies in the face of current opinion, but further studies with *kdr* strains will no doubt help in the resolution of this controversy.

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## Chapter 7

# Cloning of a Locus Associated with Cyclodiene Resistance in *Drosophila*

## A Model System in a Model Insect

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A locus associated with cyclodiene resistance has been cloned from *Drosophila*. A strain showing high levels of resistance to cyclodienes was isolated from the field and the gene mapped to the polytene subregion 66F on the left arm of chromosome three. The gene was cloned following a cosmid walk across the region and identification of several inversion breakpoints uncovering resistance. A number of cDNAs have been isolated from the locus. Sequencing of one of these showed high homology to vertebrate $GABA_A$ subunits. The susceptible phenotype has been rescued following P-element mediated germline transformation of a cosmid containing the cloned susceptible gene. The use of the cloned gene to study gene dosage, protein expression, and identification of the resistance associated mutation is discussed. Functional expression studies are described to determine the precise nature of the receptor.

In order to overcome the genetic intractability of most pest insects in which resistance is found, *Drosophila* has been proposed as a particularly efficient model insect for the study and cloning of insecticide resistance genes (*1, 2*). The aim of this chapter is to illustrate the application of this approach to the cloning of cyclodiene resistance and to show how cloned genes can be used to elucidate the basis of resistance following their isolation from *Drosophila*.

### *Drosophila* as a Model Insect for Cloning Insensitive Target Sites

*Drosophila*, alongside the housefly, has been used extensively to study metabolic resistance based on mixed function oxidases (Scott, Feyereisen, Waters, in this volume) and glutathione-S-transferases (Cochrane, in this volume). Enzymes from these metabolic systems have been purified and the genes coding for them are now becoming accessible to molecular genetics via the screening of expression libraries with antibodies raised against purified proteins. In contrast, the gene products of most target based resistance mechanisms, such as knock down resistance (*kdr*) to pyrethroids (Osborne, in this volume) or cyclodiene resistance, remain inaccessible or uncertain. The purpose of the present chapter is therefore to illustrate how *Drosophila* can be used to clone resistance genes with no previous knowledge of their product and to show, through the example of cyclodiene resistance, how this approach could be used to clone other insensitive target sites.

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One strategy for the cloning of genes in *Drosophila* relies upon the localization of the gene coding for a mutant phenotype on the detailed map of the salivary gland polytene chromosomes. Following such a localization a number of techniques exist to facilitate cloning of the gene of interest, due to the high density of cloned DNA at known positions along these chromosomes. This chapter will illustrate the isolation of a cyclodiene resistant mutant in *Drosophila*, the mapping of the gene responsible, its cloning based upon this chromosomal location, and its putative identification. Although the discussion will be based around the cloning of cyclodiene resistance, this approach would be applicable for other resistance mechanisms for which homologs could be found in *Drosophila*.

## Cyclodiene Resistance and the GABA Receptor.

Cyclodienes are thought to act at the picrotoxinin (PTX) receptor within the $GABA_A$ receptor/chloride ionophore complex (*3*). Consistent with this theory, cyclodiene resistant insects also show resistance to PTX (*4*). Ligand binding studies in strains of the German cockroach *Blatella germanica* (L.) have shown that PTX binding sites on the $GABA_A$ receptor of resistant strains possess only one tenth of the affinity for PTX of those of susceptibles, and that resistant strains may also show a reduction in the number of receptors (*5*). Recent studies with a new ligand, ethynylbicycloorthobenzoate (EBOB), have demonstrated that the binding affinity for this radioligand is reduced fourfold in a cyclodiene resistant strain of houseflies (*6*). Resistance is thus postulated to be associated with insensitivity of the cyclodiene/PTX binding site on the $GABA_A$ receptor.

Our present knowledge of GABA receptor structure and function comes from a number of cDNA's cloned from vertebrates (*7*) and one from invertebrates (*8*). These receptors are composed of several different polypeptide types that assemble to form the chloride ionophore. These polypeptide types (α, β, γ and δ) show 20-40% amino acid identity with one another. Further, each type is represented by a family of genes (e.g. β1, β2, β3) showing 60-80% identity. The composition of the different subunits forming the ionophore has been shown to affect the pharmacology of expressed vertebrate receptors. However, current understanding is insufficient to predict the location of the cyclodiene/PTX binding site and thus the possible nature of the resistance associated mutation(s).

Recently, an invertebrate $GABA_A$ receptor has been isolated from the snail *Lymnaea stagnalis* (*8*) by homology with vertebrate receptors. Genomic clones were isolated using a vertebrate $GABA_A$ β1 clone. Following the identification of several exons encoding a polypeptide with strong similarity to vertebrate β subunits, RACE (a PCR variant, rapid amplification of cDNA ends) was used to isolate a cDNA.

Insect $GABA_A$ receptors have remained uncloned due to the difficulty of obtaining suitable ligands for protein purification and the failure of vertebrate genes as heterologous probes. Thus, cloning of the cyclodiene resistance gene from *Drosophila* represented a method of not only understanding the basis of resistance but also of cloning a *putative* invertebrate GABA receptor. It should be stressed at the outset however that the cloning of the gene described here relies in no way upon previous knowledge or any assumption about the nature of the gene product. Thus, although current evidence suggests that cyclodiene resistance is associated with an insensitive $GABA_A$ receptor, we are still conducting functional expression assays to confirm that cDNAs isolated from the locus, with high amino acid similarity to vertebrate $GABA_A$ receptors, actually form GABA receptors. Throughout this text, the gene product will thus be referred to as a *putative* $GABA_A$ receptor or susceptible allele.

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## Cloning Cyclodiene Resistance

**Isolation of Cyclodiene Resistant Mutant.** High levels of insensitivity (about 4,000 fold) to dieldrin, a cyclodiene, were isolated by screening field-collected *Drosophila melanogaster*. The strain was made homozygous by 2-4 generations of selection. The single locus (*Rdl*) conferring resistance was mapped to the left arm of chromosome III. The mutant shows a semi-dominant phenotype following contact exposure to dieldrin, in common with the phenotype displayed in cyclodiene resistant insects and vertebrates. A dose of 30 µg of dieldrin applied to the inside of a glass vial discriminated between resistant homozygotes $Rdl^R/Rdl^R$ and heterozygotes $Rdl^R/Rdl^S$ (hereafter *R/R* and *R/S* respectively), whereas a dose of 0.5 µg distinguished between *R/S* and $Rdl^S/Rdl^S$ (hereafter *S/S*) flies (*9*).

**Cross-resistance, Homology and Nervous System Insensitivity.** Following repeated backcrossing to a susceptible strain, lacking elevated mixed function oxidase activity, the mutants still showed resistance to a range of cyclodienes (dieldrin, aldrin and endrin), lindane and PTX. Similar levels of resistance to these compounds were displayed as those found in other insects (*10*). Further, *Rdl* (on the left arm of chromosome three) occupies a chromosomal location homologous to that for dieldrin resistance in *Musca domestica* (chromosome IV) and *Lucilia cuprina* (chromosome V)(*11*).

In order to prove that resistance was associated with the nervous system, suction electrode recordings were taken from the peripheral nerves of transected larval central nervous systems (*12*). Treatment of nerve preparations with GABA reduced the spontaneous firing of peripheral nerves. This inhibition could be effectively reversed by the addition of dieldrin or PTX to susceptible preparations, but neither compound had an effect on resistant individuals. Thus cyclodiene resistance in *Drosophila* is present at the level of the nervous system and extends to PTX.

In summary, dieldrin resistance in *Drosophila* extends to other cyclodienes and PTX, is present at the level of the nervous system, and appears to be fully representative of cyclodiene resistance in other insects. Cyclodiene resistance is a common kind of pesticide resistance, found in at least 276 species (*13*).

**Recombinational and Deficiency Mapping.** Following recombinational mapping of the gene to approximately map unit 26 cM on the left arm of chromosome III, a number of deficiencies from this region were screened to see if they uncovered resistance. When uncovered by a deficiency, and therefore in the absence of any susceptible gene product (sensitive receptors), the resistant allele was expected to show full levels of insensitivity. Thus, for a deficiency (*Df*) uncovering resistance, *R/Df* flies will survive a dose of 30 µg dieldrin in a fashion similar to *R/R* flies. Only one deficiency within the region *Df(3L)29A6* was found to uncover resistance, whereas the overlapping deficiency *Df(3L)AC1* did not. This localized the gene to the polytene subregion 66F which is the only region of *Df(3L)29A6* not common to *Df(3L)AC1* as indicated in previous cytological mapping (*10*).

**Generation and Characterization of New Rearrangements.** In order to further localize the gene within the 66F subregion, new rearrangements uncovering the gene were generated by γ-irradiation. As flies heterozygous for resistance and any new rearrangement uncovering resistance can survive 30 µg dieldrin, new rearrangements were screened for by irradiating male *S/S* flies at 4,000 rads, crossing them to *R/R* females and screening their progeny at 30 µg dieldrin.

All the expected *R/S* progeny will die at this dose except for any progeny where the *S* allele is broken or deleted by a rearrangement uncovering resistance. New rearrangements uncovering resistance were generated at a frequency of one per 3-5,000 progeny. As this number of progeny can be screened overnight this forms a very powerful screen. All rearrangements uncovering the resistance gene were found to be lethal when crossed between themselves. This suggests that the resistance gene product is essential for formation of a viable fly.

**Chromosomal Walk and Localization of Rearrangement Breakpoints.** A chromosomal walk (*14*) was carried out in a cosmid library in order to clone the 66F subregion and identify any rearrangement breakpoints marking the location of the resistance gene. The walk was initiated from a λ genomic clone (λ121) which hybridized *in situ* to 66F1,2. Six cosmids, containing approximately 200 kb, were isolated from across the 66F sub-region (Figure 1). The last cosmids in the walk extended into 67A1,2 but failed to enter the deficiency *Df(3L)AC1*.

Single breakpoints for five of the new rearrangements generated have been located within the walk by probing Southern blots of genomic DNA from rearrangement strains with fragments from cosmid steps of the walk. One of these is from a new deficiency only visibly deleting only the cytological region 66F5. Four breakpoints are clustered within cosmid 6 (although only the first three identified are shown in Figure 1) and give two new recombinant bands on a Southern, indicating the breakpoints of either an inversion or an insertion. Cytological examination revealed that two of these breakpoints were from inversions, *In(3L)Rdl-11* and *In(3L)Rdl-20*, with one breakpoint in 66F/67A and others outside the region (*15*). These independent inversion breakpoints in cosmid 6 must therefore mark the location of the resistance gene (Figure 1).

**Isolation and sequencing of cDNA's.** In order to establish what cDNA's were being produced at the locus, a 10 kb EcoR1 fragment, spanning two of the three clustered breakpoints, was used to screen an embryonic cDNA library. A cDNA, designated NB14.1, was isolated which spanned the cluster of three rearrangement breakpoints (*15*).

Sequence analysis of this cDNA revealed one long open reading frame of 606 amino acids. The sequence of this open reading frame was used to scan DNA and protein databases, revealing highest homology with several vertebrate $GABA_A$ receptor subunits (*15*) and glycine receptors (*16*) but lacks the strychnine binding domain characteristic of the latter.

**Locus complexity.** A number of different cDNA's ranging in size from 1.0-2.5kb have been identified from the cyclodiene resistance locus. These cDNA's have been aligned based on their restriction maps and their pattern of hybridization with genomic fragments (data not shown). Due to the similarity of these different cDNAs, probing of Northern blots (unpublished data) with whole cDNAs or fragments thereof reveals a number of small transcripts (2.0-2.5kb) and one larger transcript (9kb). The status of the large transcript is uncertain but it raises the possibility that the smaller cDNAs are not full length. The purpose of the large extent of message that is presumably not coding (open reading frame of NB14.1 only 2.0kb) is unclear, but large transcripts are also found in other *Drosophila* ion channel genes such as the sodium channel locus *para*.

Many of the cDNAs we have sequenced have contained unspliced introns, thus limiting the number of candidate cDNAs. However, several cDNAs are of equal length to NB14.1 and possess similar yet different restriction maps. Experiments are therefore in progress to test the hypothesis that the locus codes for a number of different receptor subunit cDNAs by alternative splicing. This hypothesis is based on

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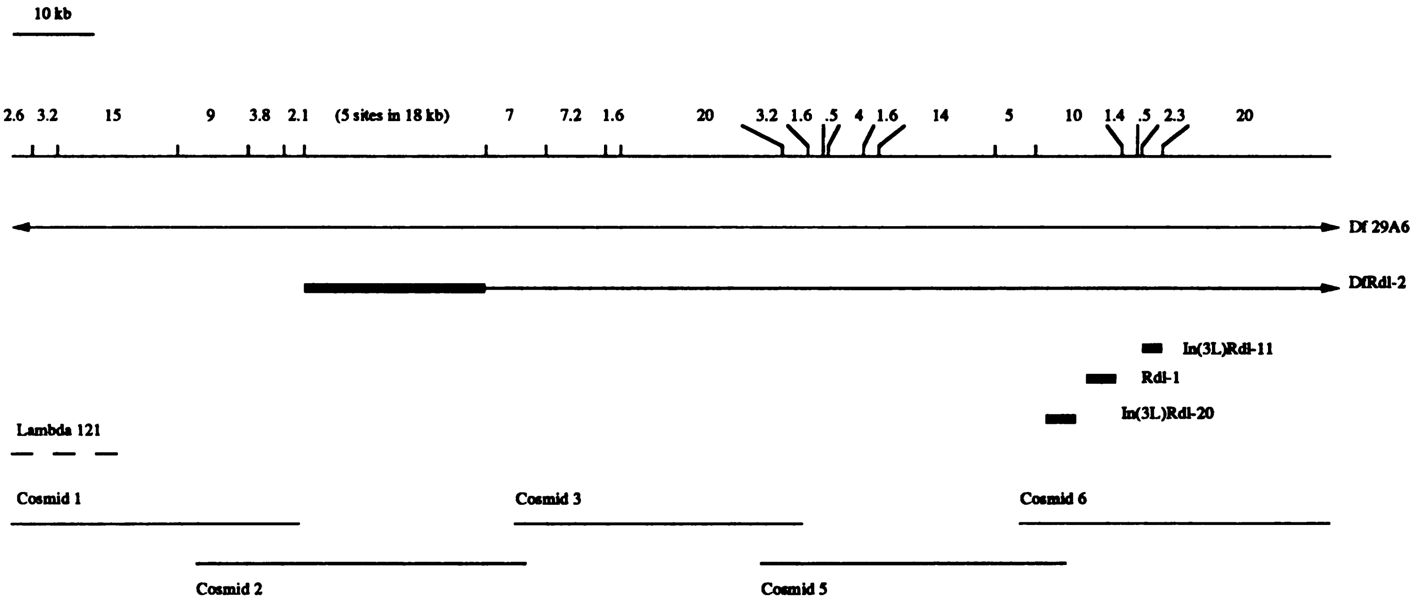
10 kb
2.6
3.2
15
9
3.8
2.1
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7
7.2
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14
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.5
2.3
20
Df 29A6
Df(Rdl-2
In(3L)Rdl-11
Rdl-1
In(3L)Rdl-20
Lambda 121
Cosmid 1
Cosmid 2
Cosmid 3
Cosmid 5
Cosmid 6

Figure 1. Chromosomal walk through the 66F polytene subregion of *Drosophila melanogaster*. The positions of the cosmids in the walk are shown below an *Eco*R1 restriction map. The solid boxes represent the positions of rearrangements uncovering the resistance gene. The location of the gene is marked by a cluster of three inversion breakpoints in cosmid 6.

the difference in restriction maps of the cDNA's, presenting the intriguing possibility that a number of different receptor subunit variants are being produced by the same locus.

## Use of the Cloned Gene to Investigate Insecticide Resistance

**Germline transformation to rescue susceptibility.** P-element mediated germline transformation was used to test if cosmid 6, the cosmid spanning the three inversion breakpoints, contained a complete functional copy of the susceptible gene. Flies heterozygous for resistance and the deficiency *Df(3L)29A6* (i.e. *R/Df*) display full levels of resistance (15% mortality after 24hrs exposure to 30 µg dieldrin), whereas all *R/S* flies die after such exposure. Therefore, *R/Df* flies with and without an inserted copy of cosmid 6 (on chromosome II) were generated to see if the *R/S* phenotype could be rescued by the insert. The resulting flies carrying the insert were susceptible (93% mortality at 24hrs) and those without were resistant (3% mortality), thus proving that cosmid 6 carries a susceptible copy of the resistance gene. The pattern of mortality of these flies over time when exposed to 30 µg dieldrin shows that the inserted copy of the gene does not fully restore the *R/S* phenotype (Figure 2). This may be due to reduced expression of the inserted gene associated with its new position in the genome.

Transformation with an overlapping cosmid (5B), displaced only 5kb distally from cosmid 6, failed to rescue susceptibility. As cosmid 5B lacks only 5kb from the proximal end of cosmid 6 and the resistance associated breakpoints occupy the opposite end of cosmid 6 (near its overlap with cosmid 5), it appears that the locus is spread across at least the 40kb of genomic DNA in cosmid 6 and that the proximal end of cosmid 6 may contain elements of the gene promoter. The full extent of the locus will be determined by mapping the 5' and 3' ends of the cDNAs to the genomic map.

**Effects of gene dosage on resistance.** The insertion of a susceptible allele onto chromosome II, independent of the native allele on chromosome III, allows for the generation of flies with varying numbers of *S* and *R* alleles and an examination of the effects of gene dosage. The $LT_{50}$'s for flies with varying numbers of *R* and *S* alleles exposed to 30 µg dieldrin are shown in Figure 3. This figure shows that, independent of the number of copies of *S* and *R*, whenever the proportions of *S* and *R* alleles are equal, flies are effectively susceptible to this dose.

It has previously been uncertain whether resistance to cyclodienes is conferred either by a change in receptor sensitivity or in receptor density (*5*). These results show that altering the number of *S* and *R* alleles does not alter susceptibility, as would be expected if resistance was associated with a change in receptor density. However, the results are consistent with the resistant allele coding for an insensitive receptor whose effect can be countered by the addition of sensitive receptors.

**Identification of resistance associated mutation.** The apparent size and complexity of the cyclodiene resistance locus complicates the identification of the resistance associated mutation. We will proceed to establish precisely which cDNA or cDNAs from the susceptible library is/are responsible for rescuing susceptibility by injecting each under control of its own promoter isolated from the genomic DNA of cosmid 6. Sequence differences between candidate cDNA's from resistant and susceptible flies will then be determined.

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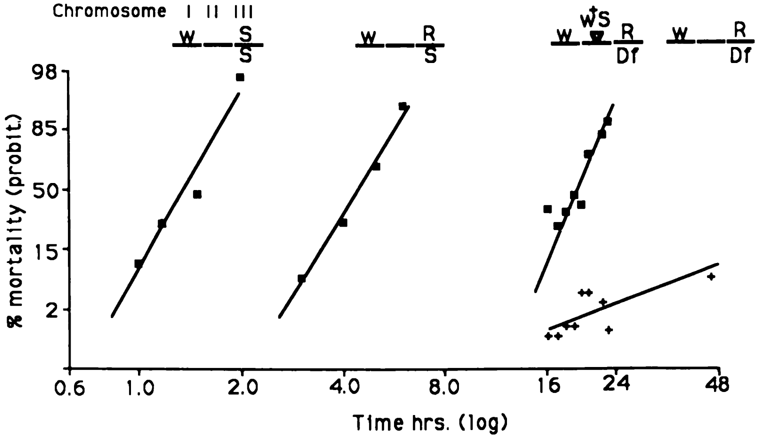

Figure 2. Time/mortality responses of *S/S*, *R/S* and *R/Df* sibs with and without an inserted copy of cosmid 6 on chromosome II. *R/Df* flies carrying an inserted copy of cosmid 6 show partial rescue of the *R/S* phenotype and have significantly greater and more rapid mortality than their *R/Df* sibs without the insert.

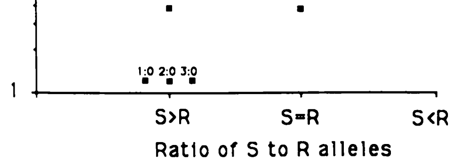

Figure 3. Relationship between the number and ratio *S* and *R* alleles and $LT_{50}$ (time in hours taken for 50% mortality to occur following exposure to 30 μg dieldrin). When *S*<*R*, flies are insensitive to this dose ($LT_{50}$'s > 24hrs). However when *S*=*R*, regardless of the numbers of each allele present, flies are sensitive to this dose ($LT_{50}$'s < 10hrs).

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## Use of cloned gene to study receptor interactions with insecticides

**Functional expression in *Xenopus* oocytes.** We have conducted functional expression studies of *Rdl* cDNAs in *Xenopus* oocytes. Weak responses to both GABA and PTX have been observed following injection with a number of cDNAs (A. Chalmers and R. ffrench-Constant unpublished). With a β subunit $GABA_A$ receptor cDNA from the snail *Lymnaea stagnalis,* functional expression of homo-oligomeric channels has been achieved. However, agonist response was dramatically increased following co-expression with a vertebrate α subunit (*8*). This demonstration that invertebrate and vertebrate subunits can form hetero-oligomeric complexes indicates that the molluscan subunit is sufficiently conserved to replace vertebrate β subunits in hetero-oligomeric channels. Further, this raises the possibility that *Rdl* cDNAs, probably coding for a single subunit type, can be co-expressed with vertebrate $GABA_A$ subunits in order to elevate levels of agonist response. We will therefore co-express the *Rdl* receptor cDNAs with vertebrate $GABA_A$ receptor α subunits in an attempt to improve the weak responses to GABA and PTX observed following expression of *Rdl* cDNAs alone (A. Chalmers and R. ffrench-Constant, unpublished data). The response of *Rdl* receptors to glycine will also be investigated.

**Production of protein from expression vectors.** The cDNA NB14.1 presumed to code for a $GABA_A$ receptor subunit is being cloned into baculoviruses for protein expression in an insect cell system. The latter system has the advantage of carrying out complete eukaryotic processing of the protein. Protein produced in this manner will be used directly in binding studies to examined the pharmacology of expressed subunits. In the long term, it is hoped to compare results from expression of susceptible and resistant proteins with binding studies carried out on fly head homogenates.

## Future Work.

The current paper has described the cloning of the locus associated with cyclodiene resistance from *Drosophila* and proof of its nature by rescue of the susceptible genotype by genetic transformation. The presumption that the cDNA isolated codes for a susceptible allele of the cyclodiene resistance gene is based upon the location of four independent inversion breakpoints, which uncover the resistant phenotype, breaking within the region coding for the cDNA. Studies have been designed to identify the genomic promoter from this locus and thus to facilitate genetic transformation of candidate cDNAs alone, as further proof that they are associated with the resistance gene.

Although functional expression studies are still in progress to confirm whether the resistance associated cDNA codes for a $GABA_A$ receptor, the availability of *Rdl* clones should now make it possible to clone homologous cyclodiene resistance associated genes from the wide range of insects in which resistance is found (*13*). Following identification of the resistance-associated mutation at the amino acid level, the nature and conservation of the mutation can then be compared between species. Examination of a number of alleles from *Drosophila* will also provide important information on the number of times the resistance mutation has arisen independently. Following the recent finding that an amplified esterase B resistance allele in *Culex pipiens* mosquitoes appears to be similar at the restriction enzyme level around the world (*17*), suggesting a single origin of the associated amplification event, further studies are needed to confirm whether similar situations exist with other resistance genes.

As well as contributing to studies on insecticide resistance, the cloning of the cyclodiene receptor allows us to further basic insect neurobiology. Through the use of functional expression systems and subunit specific antibodies, the pharmacology and distribution of *Rdl* receptor subunits will be investigated. This will allow for comparison with the pharmacology of vertebrate receptors and may allow for the rational design of insect-specific insecticides.

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## Chapter 8

# Molecular Analysis of *Methoprene-Tolerant*, a Gene in *Drosophila* Involved in Resistance to Juvenile Hormone Analog Insect Growth Regulators

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Juvenile hormone (JH) analogs offer promise for insect control. To understand insect resistance to this class of insect growth regulators, the model insect *Drosophila melanogaster* is being used to identify genes involved in resistance to methoprene. A major resistance gene is *Methoprene-tolerant* (*Met*), which results in resistance to several JH analogs by a target-site resistance mechanism involving an insensitive putative JH receptor. Several transposable element insertional alleles of *Met* have been generated, demonstrating that this type of mutagenesis can result in insecticide-resistant insects. One of these alleles is being used to clone the *Met*+ gene by transposon tagging. The potential involvement of *Met* gene homologs in pest insect resistance to JH analogs is discussed.

Chemical insecticides are effective means of controlling insect pests. However, because many insecticides have significant toxicological and environmental drawbacks, they are becoming increasingly unacceptable to the public. This awareness and attitude has lead in the past several decades to searches for novel insecticides that target specific biochemical events that are unique to insects; such insecticides pose less of a threat to other organisms. The insect growth regulators (IGR) comprise one class of insecticides to emerge from this search (1). We have been studying juvenile hormone (JH) analogs, a subclass of IGRs that disrupt development in certain insects (2-3). The most

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prominent JH analog is methoprene (isopropyl-(2E,4E)-11-methoxy-3,7,11-trimethyl-2,4-dodecadienoate), which is especially effective for mosquito and flea control (4). Since methoprene is virtually nontoxic to non-insect organisms (5), it is an attractive example of the advantage and potential of JH analog IGRs.

## Resistance to JH analogs

Since resistance is a huge problem in the chemical control of pests, a primary concern with any novel insecticide is the evolution of resistance by pest populations (6). During the early development of JH analogs, Williams suggested that insects would have difficulty evolving resistance to JH analogs. He reasoned that an insect would be unable to resist a toxic dose of a JH analog and at the same time properly regulate its endogenous JH (7). However, reports of insects resistant to methoprene soon appeared (8,9). In addition to presenting a practical problem for insect control, resistance to JH analogs begged a solution to Williams' endocrinological dilemna.

We have been interested in both of these issues brought on by JH analog resistance. In order to understand the gene(s) involved in methoprene resistance, we turned to *Drosophila melanogaster*, whose usefulness as a model insect for studying insecticide resistance has been discussed (10,11). These flies are an excellent choice for the present resistance studies because methoprene is highly effective against dipteran insects (4), including *Drosophila*. We were interested in a mutant gene(s) that would produce high resistance, outside the range of variation that has been measured in different strains of *Drosophila* (12). Roush and McKenzie (13) have argued that resistance that arises in the field is usually monogenic because selection occurs outside, not within, the naturally occurring variation. Although their argument is controversial, the identification of genes in both laboratory and field studies that result in high resistance would seem to have priority over those that result in low resistance.

**Recovery of Methoprene-tolerant mutants.** *Drosophila* males were mutagenized with ethyl methanesulfonate (EMS), and $F_1$ progeny were selected on a dose of methoprene toxic to susceptible flies. The production of polygenic resistant strains, with each gene contributing only a small amount of resistance,

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was thus avoided by this method of selection in the $F_1$ generation. A screening of several thousand progeny resulted in the isolation of a strain whose resistance was due to a gene that we have termed *Methoprene-tolerant* (*Met*). To date we have isolated 8 alleles of *Met* by EMS, X-ray, and P-element mutagenesis by the screening procedure previously described (14).

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The ease of genetic manipulation has allowed a detailed genetic characterization of *Met* (15). It was located on the X-chromosome by recombinational mapping to a region which fortuitously is rich in previously isolated deficiency chromosomes. These are chromosomes having deleted DNA, the boundaries of which can be defined by the cytological bands missing in polytene chromosome squashes. These deficiency chromosomes have allowed a detailed cytogenetic localization of the *Met* gene. This was done by first making each deficiency chromosome heterozygous with an X-chromosome carrying *Met*. These flies were then tested for resistance to a relatively high methoprene dose; if resistant, then the deficiency chromosome is missing part or all of the *Met*$^+$ gene. In this way a systematic analysis of deficiency chromosomes demonstrated that *Met* is located in the deleted region common to these deficiencies, 10C2-10D2 (Table I). This is a region containing about 10 of the approximately 5000 chromosomal bands in the *Drosophila* genome; therefore, *Met* has been localized in this manner to a precise region of the chromosome. Knowledge of the map position will facilitate molecular cloning of the gene.

When *Met* flies were tested for resistance to other classes of insecticides, no cross-resistance was detected (15). Therefore, the gene does not control some general resistance mechanism, such as increased metabolism of xenobiotics. Although JH analogs other than methoprene have not been systematically evaluated with regard to *Met* resistance, those that have been examined demonstrated that *Met* resistance extends to other JH analogs, S-11383 (18), and S-71639 (Wilson, unpublished results) as well as to JH III, one of the two naturally occurring JH hormones in *Drosophila melanogaster* (19-21). Therefore, it appears that this gene will be important for resistance to many or all of the JH analogs.

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**Table I. Cytogenetic localization of *Met* by deficiency chromosome mapping**

| Deficiency | Cytology of Deletion | Methoprene Resistance as *Met*/Df | Reference for Cytology |
|---|---|---|---|
| Df(1)m259-4 | 10C2 to 10E2 | + | 16 |
| Df(1)m-13 | 10B6-8 to 11A | + | 17 |
| Df(1)N71 | 10B5 to 10D4 | + | 16 |
| Df(1)HA85 | 10B-C to 10F | + | 16 |
| Df(1)DA622 | 10B8 to 10D2 | + | 17 |
| Df(1)KA6 | 10E1 to 11A7 | - | 16 |

Methoprene resistance was determined by survival on a dose of 0.01 µl methoprene per food vial. Neither *Met*$^+$ nor *Met*$^+$/*Met* flies survive this dose of methoprene. Results with several of these deficiencies were reported in Ref. 15.

**Biochemistry of *Met* Resistance.** To understand the mechanism of *Met* resistance, we turned to biochemical studies using radiolabelled JH III (22). Various mechanisms of insecticide resistance, including retarded cuticular penetration, tissue sequestration, and enhanced excretion or metabolism (23) were systematically analyzed and shown to be similar between *Met* and wild-type (24). However, when target site resistance (25) due to an altered cytosolic JH binding protein was examined in *Met* flies, the binding affinity ($K_D$) was found to be lower by an order of magnitude (24). We believe that this JH binding protein is a JH receptor (26). Alteration of JH receptor binding characteristics was confirmed for two other alleles of *Met* that have different genetic backgrounds. In addition, examination of *Met*/deficiency stocks indicated that the altered receptor in the *Met* strain results from the *Met* gene (or one that is very closely linked) instead of some unrelated gene in the background genome (24). In summary, we believe that the resistance of *Met* can be explained by an altered putative JH receptor that binds JH or JH analogs with less affinity than does the receptor from wild-type. Thus, the toxic effect of JH analogs is lowered because a higher dose is necessary to cause the pathological effect within the cell.

These results suggest that methoprene should also bind with lowered affinity in *Met* cytosolic extracts. This experiment was carried out using radiolabelled methoprene synthesized by G.

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Prestwich, Stony Brook, N.Y. The affinity for methoprene binding was found to be much lower than for JH III, and the relatively large amount of nonspecific binding obscured the specific binding. Therefore, we were unable to directly evaluate methoprene binding. However, methoprene binding has been indirectly evaluated by competition experiments. In extracts of *Drosophila* (26) as well as other insects (27), methoprene has been shown to be a poor competitor for binding by the natural JH. Palli et al (28) have presented evidence that in *Manduca* methoprene binds to a cytosolic binding protein separate from the one that binds JH I. If a similar situation occurs in *Drosophila*, then the separate proteins must share a common subunit that is altered in *Met* flies, since *Met* as a single gene mutation results in resistance to JH as well as methoprene.

**Strategies for Cloning the Met Gene.** For a number of reasons it would be of interest to clone the *Met* gene: (i) to determine if any sequence homology to another gene(s) of known function exists, (ii) to use a fragment of the sequence as a probe to examine temporal and tissue-specific expression of the gene, (iii) to express the gene in an expression vector and directly examine JH binding by the expressed protein, and (iv) to use a *Met* probe to isolate the homologous gene from other insects, especially pest insects such as mosquitoes.

In *Drosophila* there are several strategies for gene cloning. The simplest and most direct is to use a homologous gene from another organism as a probe in a *Drosophila* gene library. However, since the identify of the *Met* gene is unknown, this option is not feasible. Another method is called "chromosomal walking" (29), which relies on the use of a cloned gene from a nearby cytogenetic location as a probe to isolate overlapping clones from a gene library until the gene of interest is reached. Overlapping clones in the cytogenetic location of *Met*, 10C-D, have not been identified (30), and the nearest gene to this region (31) is still located an unknown molecular distance from *Met*. Therefore, a considerable walk may be involved, and repetitive DNA sequences have been reported in the 10C region (31). Repetitive DNA can slow a walk to a crawl. Overall, chromosomal walking, while feasible, presents uncertainty.

A third method is termed transposon-tagging (32). In this method a transposable element is genetically inserted into or very near the gene of interest. This event is recognized by the appearance of a mutant phenotype signalling the occurrence of a gene mutation, which in this case would be an insertional allele. A library is then made from the DNA from the insertional allele, and the gene or a portion of it is recovered by probing the library with a transposable element probe. Since transposable elements belonging to the P-element family can be readily mobilized in *Drosophila* by crosses resulting in hybrid dysgenesis (33), P elements have been utilized for transposon-tagging in our work.

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**Generation of Insertional Alleles of Met.** Movement of P elements was initiated by crossing P-cytotype males with females having the M cytotype (33). Both of these strains were methoprene-susceptible. Such a cross apparently dilutes an inhibitor of transposition in the progeny, allowing P-element movement within the germ cells. Male progeny were then crossed to females carrying *Met*$^3$, a strong EMS-induced allele of *Met*, and their progeny were raised on 0.24 µg/ml methoprene incorporated into *Drosophila* Instant Food (Carolina Biological). Male progeny from this cross were *Met*$^3$ in genotype, thus providing a positive control for survival on this high dose of methoprene. Nearly all females died at this methoprene concentration, indicating that the patroclinous X-chromosome carried *Met+*, unchanged from the parental generation. A small percentage of females survived, and their progeny were retested; most of the non-*Met*$^3$ progeny failed to survive the retest, indicting that they were false-positives. However, the result of screening several thousand chromosomes was the recovery of two lines that survived repeated retests. By complementation tests and appropriate crosses with deficiency chromosomes, these lines were determined to be *Met* alleles (Table II). They were given the experimental allele designations of *Met*$^{A3}$ and *Met*$^{K17}$. In order to carry stocks of each allele, the movement of P elements in each was stabilized by repeated backcrosses of each line with an attached-X stock having many P elements and a high level of transposition repressor (33).

It was necessary to establish that each of these alleles was indeed a P-element insertional allele instead of some other type of mutation. This was done in two ways: (i) establishing that each is unstable, a characteristic of P-element insertional alleles (33), and (ii) establishing the presence of a P element in the cytogenetic location of *Met* by *in situ* hybridization to salivary gland chromosome squashes from larvae carrying each of the alleles.

**Table II. Survival of P-Element Alleles and Revertants on Methoprene Food**

| Genotype | Mutant Type | % Survival Methoprene Dosage (µl/vial) | | | |
|---|---|---|---|---|---|
| | | 0.0 | 0.005 | 0.01 | 0.05 |
| *Met*$^{A3}$ /Y | P element | 73 | 60 | 53 | 36 |
| *Met*$^{K17}$/Y | P element | 95 | 96 | 91 | 13 |
| 141/Y | Revertant | 76 | 0 | 0 | 0 |
| K1/Y | Revertant | 83 | 0 | 0 | 0 |
| Oregon-RC | wild-type | 87 | 0 | 0 | 0 |

The survival values for Oregon-RC are given for comparison. Since the P-element alleles are maintained as males with attached-X females having a P-cytotype (33), only males carry a *Met* mutation. Therefore, the survival values above are for males for each of the strains listed.

First, the instability of each allele was evaluated by crossing *Met*$^{A3}$ and *Met*$^{K17}$ males with an M-strain to encourage transposition of the P-element. If these alleles are indeed insertional alleles, then two types of progeny will be generated in low frequency: (i) susceptible individuals, resulting from precise excision of the P-element and regeneration of the *Met*+ gene, and (ii) lethal revertants, resulting from imprecise excision of the P element, during which flanking DNA carrying a vital gene is concurrently lost upon excision of the P-element.

The outline of the crossing scheme is shown below. *FM7* refers to an X-chromosomal balancer chromosome that is marked with the *Bar* eye mutation, and Y refers to the Y-chromosome, and in some crosses this chromosome carried the $y^+$ marker. *C(1)DX, y f* is an attached-X stock marked with the *yellow* (*y*) and *forked* (*f*) mutations. The chromosome carrying *Met*$^{A3}$ also carries the marker genes *y* and *vermilion* (*v*). *Met*$^{R}$ refers to a potential revertant, which was distinguished as either lethal or methoprene susceptible by cross #1 or #2, respectively.

Using this mating scheme, we found that both classes of revertants were generated. *Met*$^{A3}$ gave a lethal reversion

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frequency of 2% (N=200), and a methoprene-susceptible reversion frequency of 1% (N=300). *Met*$^{K17}$ gave a somewhat

$y\ v\ Met^{A3}/Y$ ♂ X $C(1)DX, y\ f/y^{+}Y$ ♀
↙
$y\ v\ Met^{A3}/y^{+}Y$ ♂ X $FM7/FM7$ ♀
(dysgenic) ↙
$y\ v\ Met^{R}/FM7$ ♀ X $FM7/y^{+}Y$ ♂(single pair mating)

cross #1 (regular food) ↙ | cross #2 (raised on methoprene) ↘
absence of non-*Bar* males (lethal revertant) | absence of non-*Bar* males (susceptible revertant)

lower lethal reversion frequency of 1.5% (N=200) and methoprene-susceptible reversion frequency of 0.5% (N=200). The absence of methoprene resistance for two susceptible revertants, stock 141 (*Met*$^{A3}$ revertant) and stock K1 (*Met*$^{K17}$ revertant) is shown in Table II. As expected, neither class of revertant could be generated from our original EMS-induced *Met* allele (N=200). Therefore, revertants are not characteristic of *Met* mutations in general, but only of the P-element insertional alleles. Although our reversion studies were not extensive, these frequencies that we measured are comparable to those found for other *Drosophila* P-element insertional alleles that have been studied (34). In summary, it appears that both *Met*$^{A3}$ and *Met*$^{K17}$ are unstable, a characteristic of transposable element insertional alleles.

Finally, the presence of a P-element within the 10C-D region of the X-chromosome was verified by *in situ* hybridization to salivary gland chromosomes from one of the P-element alleles. A band of silver grains can be seen in this region of *Met*$^{A3}$ (Figure 1). Therefore, it appears likely that both alleles are P-element insertional alleles, and that *Met*$^{A3}$ is suitable for library construction.

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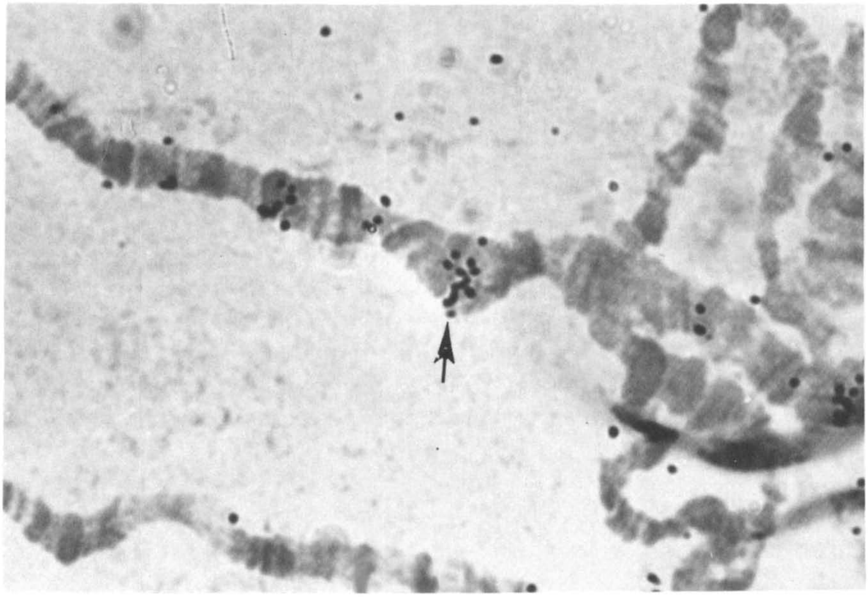

Figure 1. *In situ* hybridization of [$^3$H]thymidine-labeled P-element DNA (from plasmid pπ25.1) to the X-chromosome from a salivary gland squash of a *Met*$^{A3}$ male. The arrow indicates the band of silver grains in the 10C-D region.

**Construction of a *Met$^{A3}$* genomic library.** DNA was prepared from flies carrying the *Met$^{A3}$* allele, partially cut with *Sau*3A, and sized on a salt gradient following centrifugation. DNA corresponding to 12-16 kb size fragments was taken from the gradient and ligated into the EMBL3 phage vector. After packaging, the phage were plated out, and lifts from the plates were probed with a P-element probe, pπ25.1, labelled with $^{32}P$ by nick-translation. Approximately ninety colonies that were positive were picked, plaque purified, and stored in glycerol at -20 C.

Since the *Met$^{A3}$* genome has P-elements at other sites in addition to the insertion in 10C-D, it will be necessary to identify a clone that originated from 10C-D. Such a clone can be distinguished from the others in the collection by *in situ* hybridization. Therefore, DNA mini-preps are being prepared from each plaque and sequentially hybridized to polytene chromosomes. Since each plaque contains P-element sequence, the chromosome preparations must be from an M-strain of *Drosophila* which lacks this sequence. In this way we hope to identify DNA derived from a portion of the *Met* gene. Using this DNA as a probe, a library from a methoprene-susceptible strain can be screened to identify the homologous sequence, and the entire *Met$^{+}$* gene can be cloned by a short bidirectional chromosomal walk.

## Conclusions

**Utility of *Drosophila* for Resistance Gene Detection.** In this work we have shown the usefulness of *Drosophila* for an evaluation of resistance to JH analogs and detection of the gene(s) involved. This methodology should be applicable to any insecticide. By standard mutagenesis procedures and screening of progeny, one or more resistance genes can be identified. In this way resistance can be examined *before* an insecticide is field-tested. Once a resistant mutant is detected, the mutant can be studied genetically and biochemically, and the gene can be molecularly cloned. This type of preliminary resistance estimation might minimize surprises, such as the recent detection of resistance to *Bacillus thuringiensis* (35), as well as suggest strategies to manage the anticipated resistance when it develops in the field.

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**Transposable Element Movement as a Factor Initiating Resistance in the Field.** Several recent studies have expanded our knowledge of the genetic changes that lead to resistance in the field. For example, gene amplification leading to organophosphorus insecticide resistance in mosquito populations has been detected (36). However, we still have little knowledge of the molecular events leading to resistance. The involvement of transposable elements in producing genetic change must certainly be considered, especially since the movement of some of them, especially P elements, is very active (33). Indeed, at least in *Drosophila*, many spontaneous mutations that have arisen over the years of *Drosophila* research appear to be due to transposable element insertional mutagenesis (37). Recently, a field strain of *Drosophila*, 91R, that was selected for DDT resistance has been shown to have enhanced levels of cytochrome P450. This gene has been sequenced from both 91R and control flies, and a remnant of a transposable element appears to be associated with the resistance (Waters et al, this book).

Although our results are strictly laboratory generated, we have demonstrated that resistance to an insecticide can be caused by transposable element insertional mutagenesis. There is no reason why movement of transposable elements in field populations of insects could not result in resistant insects.

**Do other Insects have a *Met* Gene?** Although it is possible that the *Met* gene is unique to *Drosophila* and therefore would be unimportant to pest insects for resistance to JH analogs, we do not believe that this to be the case. First, this type of resistance mechanism--target-site resistance--is a widespread mechanism that has now been documented for a variety of insecticides (25). Second, JH analogs affect insects in a manner similar to that produced by topical application of an excessive amount of the natural hormone. This implies that JH analogs are affecting a physiological step(s) in the JH endocrine system. Our results indicate that the *Met*+ gene encodes a putative JH receptor or at least is involved in a stoichiometric manner with JH reception (24). Since JH is widespread in Insecta and appears to be a conserved hormone (38), there is no reason why the receptor, and therefore the *Met*+ gene, would not also be conserved. Although the actual sequence of the *Met*+ gene may show variation within Insecta, the function of the *Met*+ gene--binding of JH and JH analogs--is likely to remain the same. This function can be changed by mutation of the gene, which might result in poorer binding of JH analogs and thus resistance of the insect to toxic doses. It therefore seems likely that genes homologous to *Met* will be important in resistance to JH analogs in a variety of insects.

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## Chapter 9

# Cytochrome P450 Monooxygenase Genes in Oligophagous Lepidoptera

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Cytochrome P-450 monoxygenases are involved in metabolism of hostplant allelochemicals by Lepidoptera. Although P450 monoxygenases in polyphagous species are broadly substrate-specific, lepidopterans with narrow host ranges appear to have specialized P450 activities. *Papilio polyxenes*, the black swallowtail, is a specialist on furanocoumarin-containing plants in the Apiaceae and Rutaceae. We have cloned and sequenced cDNAs coding for a P450 that is specifically induced by xanthotoxin, a linear furanocoumarin common in many black swallowtail hostplants. Although the two *CYP6B1* cDNA clones contain restriction site polymorphisms, the proteins encoded by them are 98.2% identical, suggesting that these clones represent allelic variants at a single locus. Northern analysis of mRNA from related *Papilio* species with these cDNAs reveals that *P. brevicauda*, a closely related species that is also a specialist on Apiaceae, contains an mRNA crossreactive with *CYP6B1*. mRNAs were not detectable on Northerns at high stringency from more distantly related species (*P. cresphontes* and *P. glaucus*) that have inducible xanothotoxin metabolism but that feed on different plant hosts (Rutaceae and a variety of tree species) with different furanocoumarin contents. The structure and function of P450s involved in metabolism of host chemicals may thus reflect evolutionary associations with these host chemicals.

Virtually every organism at some point during its life is exposed to environmental toxins. Accordingly, most organisms are equipped with systems for detoxifying and excreting potentially toxic environmental chemicals (*1*,*2*). The metabolism of xenobiotics generally occurs in two phases. Phase I metabolism, or functionalization, consists of enzymatic alteration of the chemical structure of the toxin by introduction of a functional group. Phase II metabolism, or conjugation, involves attachment of the altered toxin to a carrier substance to export it out of the body (*3*). Cytochrome P450 monooxygenases, which effect a wide range of oxidative transformations, represent the most widespread of the Phase I enzymes and mediate reactions such as C, N, and S-hydroxylations, demethylations, deethylations, deaminations, and dehalogenations. These types of oxidative

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reactions typically convert lipophilic toxins into more hydrophilic, and hence excretable, metabolites.

Primary amino acid sequence comparisons of more than one hundred genes coding for mammalian, yeast, bacterial, plant and insect P450s have produced a phylogenetic tree that reflects the presumptive evolutionary relationships within the P450 gene superfamily (*1*). P450 genes within the same family generally share more than 40% amino acid identity; subfamilies within families share more than 59% amino acid identity. Twenty-seven P450 families have been described; insect P450s sequenced to date have been placed in family *CYP6* (*4,5*) and *CYP4* (*6*). Phylogenetic analysis indicates that P450 genes, particularly *CYP2*, proliferated approximately 400 million years ago, coincident with the appearance of terrestrial herbivores and plants. Gonzalez and Nebert (*1*) have suggested that the diversity of plant secondary substances encountered by herbivores may have led to the diversification of *CYP2*, a vertebrate family. Many more insect P450s must be characterized in order to determine whether a similar proliferation of P450s occurred in the Insecta as land plants were colonized.

The metabolism of plant products by P450 monooxygenases in herbivorous animals, either vertebrate or invertebrate, has been poorly characterized. In insects, particularly Lepidoptera, xenobiotic-metabolizing P450 monooxygenases are generally regarded as broadly substrate-specific enzymes (*3*). Their role in the metabolism of xenobiotics was first discovered in connection with insecticide resistance because many P450s are involved in the metabolism of synthetic organic insecticides. Krieger et al. (*7*), who first compared monooxygenase activities in a broad cross-section of the Lepidoptera, suggested that these enzymes exist in midgut tissue for the metabolism of potentially toxic compounds encountered in hostplants. They hypothesized that monooxygenase activity levels are elevated in polyphagous species compared to oligophagous species because polyphagous insects encounter a broader range of potentially toxic substrates in their many chemically distinct hostplants than do oligophagous insects, which feed on a narrower range of more chemically uniform plants.

Since 1971, there has been increasing evidence that polyphagy and high P450 activity are not absolutely linked (*8,9*). One possible alternative explanation for the findings of Krieger et al. (*7*) is that they measured P450 activity directed against aldrin, a synthetic organic insecticide. It is likely that these synthetic compounds, relative novelties in the evolutionary history of insects *(10)*, are metabolized by P450s that are not particularly substrate-specific and that P450s with broad substrate specificity are most abundant in insects that encounter a wide range of hostplant metabolites. Thus, the extensive use of selective assays of P450 activity against synthetic substrates has led to the widespread supposition that xenobiotic-metabolizing P450s in insects are broadly substrate-specific. This belief has been further substantiated by the fact that xenobiotic-metabolizing P450 structure and function relationships have been defined primarily in mammals that are either highly omnivorous (such as the rat) or broadly polyphagous (such as the rabbit). Even the most polyphagous caterpillar is unlikely to encounter as many plant species over the course of its lifetime as a rabbit potentially encounters while grazing over the course of a single day. Probabilities of encountering particular allelochemicals, as well as amounts actually ingested once encountered, are undoubtedly factors influencing the evolution of substrate-specificity in xenobiotic-metabolizing P450s.

## P450-Mediated Metabolism of Furanocoumarins

Only recently has P450-mediated metabolism of naturally occurring constituents of insect hostplants received experimental attention. One group of plant secondary compounds that has been widely studied for the last decade are the furanocoumarins.

These naturally occurring phenolic derivatives occur in fewer than a dozen plant families and are most diverse and widely distributed in the Rutaceae and Apiaceae (*11*). Two main classes of furanocoumarins, linear and angular, differ in their biosynthetic origin. Linear furanocoumarins, in which the furan ring is attached to the coumarin nucleus at the 6 and 7 positions, are widespread in both plant families. Angular furanocoumarins, in which the furan ring is attached at the 7 and 8 positions, are restricted to a few genera of Apiaceae (Figure 1).

Furanocoumarins are broadly biocidal (*11*). In insects, furanocoumarin toxicity is manifested in the forms of feeding deterrency (*12,13*), molting impairment (*14*), developmental delay (*15,16*) and death (*15,17,18*).

Toxicity notwithstanding, a number of Lepidopterans feed exclusively on furanocoumarin-containing plants and appear to be metabolically specialized for efficient furanocoumarin detoxification. Several furanocoumarins are known to be metabolized by P450 monooxygenases (*19-22*). Three aspects of the P450 metabolism of furanocoumarins in these species suggest that, in contrast to the evolution of P450s responsible for metabolism of synthetic organic insecticides, these furanocoumarin-metabolic P450s may have evolved for specialized functions subject to selection by host chemicals. First, a comparison of furanocoumarin metabolism in generalists, which occasionally encounter furanocoumarin-containing plants, and in specialists, which inevitably encounter larger amounts of furanocoumarins in their hosts, demonstrates that specialists metabolize furanocoumarins significantly faster than do generalists. Metabolism of xanthotoxin, a linear furanocoumarin, has been documented in several generalized noctuids (Table I), which feed on a wide range of host families (in some cases, greater than 100 different host species).

**Table I. P450-Mediated Metabolism of Xanthotoxin by Midgut Microsomes of Lepidopterous Larvae**

| Species | Enzyme Activity (nmol/min/mg protein) | | Reference |
|---|---|---|---|
| | noninduced | induced | |
| *Depressaria pastinacella* | 21.0 | 31.0 | (*21*) |
| *Papilio polyxenes* | 1.1 | 8.33 | (22) |
| *Spodoptera frugiperda* | 0.11 | not done | (*41*) |
| *Trichoplusia ni* | 0.15 | 0.15 | (*42*) |

Furanocoumarin-containing plants are never frequent, much less major, hosts for any of these species -- all appear to utilize furanocoumarin-containing plants on an incidental basis (*Helicoverpa zea* (*23*), *Spodoptera frugiperda* (*19,20*), *Trichoplusia ni* (*24*)). Levels of xanthotoxin metabolism in these generalists are 10 to 80-fold lower than in oligophagous species that are effectively restricted to furanocoumarin-containing hosts. One such species, ***Papilio polyxenes*** (black swallowtail), feeds on foliage of more than a dozen apiaceous genera, all of which contain furanocoumarins

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(*25*). *Depressaria pastinacella* (parsnip webworm), a caterpillar restricted to fruits and flowers of two genera of Apiaceae, also has extremely active systems for furanocoumarin metabolism (*21*). This species, which encounters furanocoumarin levels in plant reproductive tissues up to ten-fold higher than in foliage, is capable of metabolizing xanthotoxin up to 30 times faster than black swallowtails and up to 300 times faster than the more polyphagous noctuids. Thus, the levels of these xanthotoxin-metabolic P450 activities run completely counter to the prediction of Krieger et al. (*7*), which suggests that the levels of monooxygenase activity should be lowest in the most specialized insects.

The second aspect of P450 metabolism of furanocoumarins that suggests that some insect P450s have evolved for specialized functions is that, within a species, the rates of P450 metabolism vary with furanocoumarin structure. Linear furanocoumarins are metabolized significantly faster in at least two species, the specialists *P. polyxenes* and *D. pastinacella*, than are angular furanocoumarins (Table II). This difference in activity is consistent with the fact that these insects encounter angular furanocoumarins far less predictability and in far lower concentrations than they encounter linear furanocoumarins. For example, only two of the 12 host genera of *P. polyxenes* are reported to contain angular furanocoumarins (*16*); although all genotypes of the principal host plant *Pastinaca sativa* for *D. pastinacella* produce linear furanocoumarins, some genotypes lack angular furanocoumarins altogether (A. Zangerl and J. Nitao, pers. commun.).

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**Table II. Comparison of P450-Mediated Metabolism of Linear and Angular Furanocoumarin by Lepidoptera**

| Species | nmoles metabolized/min/g fresh weight larva | | Reference |
|---|---|---|---|
| | linear | angular | |
| *Papilio polyxenes* | 8.1 - 9.9 | 2.8 | (*43*) |
| *Depressaria pastinacella* | 19.2 -26.0 | 10.9 | (*44*) |

Finally, in at least some specialists, P450-mediated furanocoumarin metabolism is induced specifically by the furanocoumarin that the P450 detoxifies (*21*,*22*). Efforts to induce xanthotoxin metabolism with general P450 inducers in *P. polyxenes* were unsuccessful (*9*). In contrast, these general inducers are capable of inducing metabolism of many synthetic substrates in omnivorous species such as the house fly, *Musca domestica* (*26*,*27*). In the specialists, the degree of inducibility may also reflect the ecological variability of the presumptive substrate in the insect host. Black swallowtails feed on both foliage and flowers of a broad range of apiaceous plants, many of which contain only trace amounts of furanocoumarins and others of which contain more than 1% dry weight furanocoumarins (*25*). These insects are capable of inducing xanthotoxin-metabolic activity up to seven-fold (*22*). In contrast, *D. pastinacella* feeds almost exclusively on reproductive parts of *Pastinaca sativa*, which are consistently high in furanocoumarin content. These insects, which rarely if ever feed on plant tissues low in furanocoumarin content, have a high constitutive xanthotoxin-metabolic activity that is only 0.5-fold inducible by xanthotoxin (*21*).

The evolution of specialization in xenobiotic-metabolizing P450 activities likely involves changes in both protein structure and regulation of expression. Presumably, the primary structure of specialized P450s evolves to yield greater substrate affinity and turnover rate. Greater induction of enzymatic activity in response to the substrate probably arises as well. Both transcriptional and post-transcriptional mechanisms of induction have been reported for P450s (*28*). Post-transcriptional mechanisms include increased stabilization of mRNA or protein. Transcriptional induction has been well characterized for one group of xenobiotic-metabolizing P450s, the *CYP1* family. Transcription of these P450s is initiated following the binding of inducers to the cytosolic aromatic hydrocarbon (Ah) receptor and the subsequent binding of the receptor-ligand complex to regulatory DNA sequences. Receptors for other xenobiotics have proven elusive. The Ah receptor has been shown to occur in *Drosophila melanogaster* (*29*) and it will be of great interest to determine if more specialized receptors for hostplant defensive compounds have evolved in oligophagous insect herbivores.

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## Cloning of *CYP6B1* cDNA from *P. polyxenes*

To investigate the evolution of P450s important in the detoxification of plant secondary metabolites, we have been examining the molecular basis of P450-mediated xanthotoxin metabolism in *Papilio polyxenes*. This species feeds exclusively on furanocoumarin-containing species in the Apiaceae and Rutaceae (*25*). The metabolism of the linear furanocoumarin xanthotoxin in this species is inducible up to seven-fold by xanthotoxin and occurs concomitantly with the appearance of an approximately 57 kD polypeptide (*9,22*). To clone cDNAs encoding a xanthotoxin-inducible P450, the 57 kD inducible protein was gel purified from larval midgut microsomes and partially sequenced. Fully degenerate primers were designed to sequences of six amino acids at the N-terminus and seven amino acids from an internal fragment (Figure 2) (*5*). The internal oligonucleotide was used to prime reverse transcription of poly (A)+ RNA isolated from induced larval midguts. The N-terminal primer was then added for PCR amplification, resulting in a single product of 1.2 kb. Partial sequence analysis of the PCR product indicated that it encoded a cytochrome P450 and, on Northern blots, it crossreacted with midgut mRNAs of 1.6 kb highly inducible by xanthotoxin (*30*).

Full length cDNAs were obtained by screening a cDNA library prepared from xanthotoxin-induced larval midgut poly (A)+ RNA with the 1.2 kb PCR product. Two clones, each 1.6 kb long and distinguishable by the presence (*CYP6B1.1*) or absence (*CYP6B1.2*) of internal XhoI and HindIII sites, were isolated and sequenced (*5,31*) The clones encode proteins of 57 kD that are 98.2% identical and contain the sequence
F--G---C-G (Figure 3). This sequence, which contains the cysteine that forms a ligand to the heme iron in the P450 catalytic site, is highly conserved among P450 proteins (*28*). Recent analysis of genomic DNA has confirmed that the clones encode alleles of a single locus (*31*). The xanthotoxin-inducible P450 is 32.1% identical in amino acid sequence to *CYP6A1* from the house fly (*4*). Despite the fact that this level of identity is less than the 40% required for inclusion in the same family, the xanthotoxin-inducible P450 has nonetheless been designated *CYP6B1*.

On Northern blots, *CYP6B1* crossreacts with mRNAs highly induced when black swallowtail larvae are fed xanthotoxin-supplemented parsley or untreated parsnip, a host with high endogenous levels of furanocoumarins (Figure 3). Although the xanthotoxin content of the parsnip foliage used in this experiment was considerably lower than that of the treated parsley, 0.2% of fresh weight (*30*),

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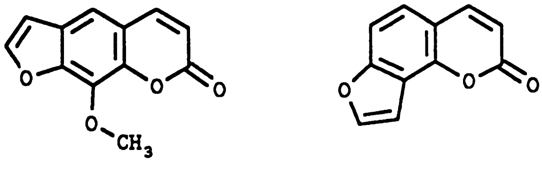
O
O
O
O
CH3
O
O
O

xanthotoxin angelicin

Figure 1. Furanocoumarin Structures

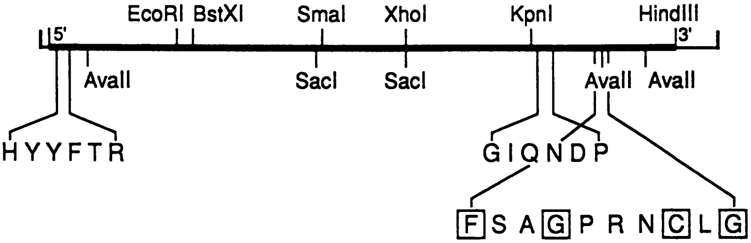
EcoRI BstXI
SmaI
XhoI
KpnI
HindIII
5'
3'
AvaII
SacI
SacI
AvaII AvaII
HYYFTR
GIQNDP
F S A G P R N C L G

Figure 2. CYP6B1.1 cDNA

The *CYP6B1.1* cDNA is drawn with the N-terminal and internal amino acids used to generate oligonucleotide primers for PCR amplification shown below the restriction map. The conserved amino acids present near the heme binding site in P450s are boxed.

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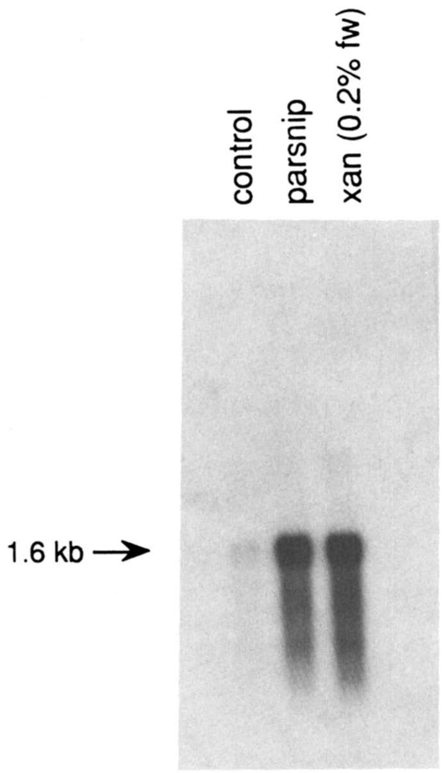

**Figure 3. Northern Analysis**

Northern blot of black swallowtail midgut poly(A)+ RNA probed with 1.6-kb insert of clone *CYP6B1.2*. Final instar larvae were reared for 2 days on a control diet consisting of acetone-treated parsley, a diet of untreated parsnip or a diet of parsley treated with 0.2% (fresh weight) xanthotoxin. Two micrograms of each poly(A)+ RNA sample was electrophoresed on a 1% agarose-formaldehyde gel in MOPS buffer, transferred to Zetaprobe membrane and probed at high stringency with the $^{32}$P-labeled 1.6kb insert of *CYP6B1.2*.

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CYP6B1 mRNAs were induced to similarly high levels in larvae fed on both diets (Figure 3). Xanthotoxin is generally the most abundant furanocoumarin in parsnip foliage, although other furanocoumarins, both linear and angular, are also present (*17*). Additional research is necessary to quantify the effects of the various furanocoumarins, and possibly other secondary metabolites, in parsnip that contribute to CYP6B1 induction.

## Immunochemistry of Xanthotoxin-Inducible P450s in *P. polyxenes*

Monoclonal antibodies to CYP6B1 have been generated and characterized (*32*). Three BALB/c mice were immunized with the 57 kD inducible protein and primary antisera were screened by immunoblots with xanthotoxin-induced microsomal protein. The mouse spleen cells were subsequently fused with SP2/O-Ag 14 myeloma cells and the resulting cell lines were screened and subcloned.

One of the subclones, screened by immunoblot with midgut microsomal proteins, crossreacted selectively with the 57 kD xanthotoxin-inducible P450. Immunoblots with this monoclonal antibody on proteins isolated from black swallowtail larvae fed different synthetic inducers (*32*) revealed that the abundance of the xanthotoxin-inducible P450 polypeptide is unaffected by synthetic inducers previously shown to have little effect on xanthotoxin-inducible activities (*9*). The immunoblots revealed the presence of a second crossreactive P450 that is induced approximately two-fold by xanthotoxin. Further characterization of this protein is in progress.

## Evolution of Xanthotoxin-Inducible P450s in Papilionidae

Using the *CYP6B1* cDNA and monoclonal antibodies raised against the CYP6B1 protein as probes, we have begun to investigate the evolution of this P450 by examining additional species of the subfamily Papilioninae (*5,32*). *Papilio brevicauda*, like *P. polyxenes*, is a specialist on furanocoumarin-containing plants and, as a member of the *machaon* complex, is regarded as a very close relative of *P. polyxenes* (*33*). *P. cresphontes*, the giant swallowtail, feeds exclusively on furanocoumarin-containing rutaceous hosts. *P. glaucus* is the most polyphagous of all papilionids and feeds on the foliage of many tree families (*34*). Although it has been reported, albeit rarely, to feed on *Ptelea trifoliata* (hop tree), a furanocoumarin-containing rutaceous plant (*35*), the vast majority of its hosts do not contain furanocoumarins. Even though it is a close relative of the polyphagous *P. glaucus* (*36*), *P. troilus* is restricted to hosts in the Lauraceae, none of which appear to contain furanocoumarins. Papilionids outside the tribe Papilionini are not known to feed on furanocoumarins. For comparative purposes, we examined furanocoumarin metabolism in members of two of these tribes. The troidine *Battus philenor*, which is confined to hosts in the Aristolochiaceae, and the graphiine *Eurytides marcellus*, which is restricted to species in the Annonaceae, are incapable of surviving from first instar on hostplant foliage topically treated with xanthotoxin (*18*).

On Northern and Western blots, a probable orthologue to *CYP6B1* has been identified in *P. brevicauda*, the closest relative of *P. polyxenes* examined. Possible orthologues to *CYP6B1* are detectable when Northern blots of poly (A)+ RNA from two less closely related congeners of *P. polyxenes* are screened at low stringency: *P. cresphontes*, a Rutaceae specialist, and the highly polyphagous *P. glaucus*. No crossreactive mRNAs were detected in *P. troilus* or in two species from other tribes of the Papilioninae (*5*).

Rutaceae-feeding is widespread in the genus *Papilio* and it is possible that *CYP6B1* arose in ancestral *Papilio* species as this furanocoumarin-producing plant family was colonized. If, however, Rutaceae-feeding arose independently several times within *Papilio* (*37*), then *CYP6B1* and the P450s crossreactive with it in *P. cresphontes* and *P. glaucus* may have all evolved independently from a common ancestral P450 isozyme. Further characterization of P450s from *Papilio* species will shed more light on the influence of hostplant colonization events on P450 evolution.

By Northern analysis, we have also determined that *Depressaria pastinacella* (parsnip webworm), an oecophorid also specialized on the Apiaceae, does not have mRNAs crossreactive with *CYP6B1* (*5*). This finding suggests that the P450 genes encoding furanocoumarin-metabolizing proteins in webworms are unrelated to *CYP6B1*. This lack of relationship is entirely consistent with the distant phylogenetic relationship between the Oecophoridae, the microlepidopteran family to which *D. pastinacella* belongs, and the Papilionidae, the macrolepidopteran family to which *P. polyxenes* belongs (S. Passoa, pers. commun.), and indicates that distantly related insects that feed on the same plants may utilize unrelated P450s to metabolize the same hostplant defensive compounds.

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## Conclusions

In contrast with vertebrate (particularly mammalian) P450s, insect P450s have received little attention with respect to their molecular genetics and regulation (*4-6*). This lacuna in knowledge is surprising in view of the fact that the study of P450 genes and their regulation in insects, particularly oligophagous Lepidoptera, holds great promise for providing answers to many puzzling questions about the evolution of P450 genes. First of all, oligophagous insects are often exceedingly restricted in their diet--some species, for example, eat only particular parts of certain plants at particular times of the year. Thus, identifying naturally occurring P450 substrates that may act as selective agents on insect metabolism is greatly facilitated, in comparison with identifying such compounds in the diets of omnivorous or even broadly polyphagous herbivorous species. Moreover, by virtue of their hostplant specificity, oligophagous species may encounter far greater concentrations of particular toxins than do more polyphagous species. For example, the parsnip webworm, *Depressaria pastinacella*, feeds exclusively on the furanocoumarin-rich flowers and fruits of *Pastinaca sativa*; in a single day, a webworm can ingest quantities of furanocoumarins equivalent to as much as 7% of its total body weight (*38*; A. Zangerl and M.R. Berenbaum, pers. observ.). Such massive exposure to dietary toxins is likely to have a profound impact on the detoxicative metabolism of an organism.

Secondly, herbivorous insects have speciated in a spectacular fashion; there are at least 300,000 species of insects that utilize plants as a major source of nutrients (*39*). In many of these herbivorous groups, evolutionary relationships are well documented, thus providing a phylogenetic framework for reconstructing patterns of evolutionary change in P450 gene structure and function. The staggering diversity of herbivorous groups (which include taxa in at least nine orders of arthropods) (*39*) provides ample opportunities for comparative studies and for investigations of parallel or convergent evolution.

Finally, P450-mediated metabolism of xenobiotics by herbivorous insects is of tremendous economic importance inasmuch as it is responsible at least in part for the resistance of agronomically destructive pests to synthetic organic insecticides (*40*). The development of new and effective insecticides is seriously hampered by the rapid evolution of resistance, often by means of P450-mediated cross-resistance to insecticides used previously, in target species. By understanding the mechanisms by which P450s can evolve to specialize or to adapt to novel substrates, insights may be gained into circumventing the evolutionary processes and thereby prolonging the useful lifetime of control chemicals in an integrated pest management program.

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## Chapter 10

# Effects of the Endophyte-Associated Alkaloid Peramine on Southern Armyworm Microsomal Cytochrome P450

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Several kinds of alkaloids are produced in plants containing endophytic fungi. The association is mutualistic (1). The fungus derives nutrients, protection, and propagation sometimes without sporulation and the plant gains protection from herbivory and in some cases increased growth rate and drought resistance (2). The alkaloids are responsible for the antiherbivory effects. Some alkaloids, e.g., peramine, are antifeedants for insects, others are toxic to insect and vertebrate herbivores. Peramine interferes with microsomal cytochrome P450 causing the carbamate insecticide carbaryl to be twice as toxic as normal to Spodoptera eridania caterpillars.

Endophytic fungi live their whole life, or all of it except for the reproductive stage, inconspicuously in the tissues of plants (3). The ecological or physiological effects of endophyte-associated alkaloids on insect herbivores are not well understood and have only been studied recently (1, 4-7).

Peramine and lolitrem B are the two major N-heterocyclic compounds in perennial ryegrass, Lolium perenne, containing the endophytic fungus Acremonium lolii (8, 9).

The toxic action of the alkaloids is unknown as are their interactions with detoxifying enzymes, in particu-

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lar, cytochrome P450. This enzyme is important in herbivores and is known to metabolize alkaloids and insecticides (10). Interactions between the alkaloids and cytochrome P450 may affect the suitability of endophyte-containing plants as food sources for herbivores or even the efficacy of synthetic insecticides. In this paper, we first give a summary of the effects of endophytes and then, we present data on the interactions between pure peramine and microsomal cytochrome P450 from *Spodoptera eridania* caterpillars.

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## Effects of endophytes

**The endophyte/grass complex.** Ascomycetes in the family Clavicipitaceae, tribe Balansiae inhabit many plant species as endophytes. As many fungi in this tribe appear to be completely endophytic, occurring only as intercellular mycelia in plant tissues, and sporulation stages have not been found, classification is not without controversy (1, 3). Many of these fungi appear to be transmitted exclusively by hyphae in seeds to the next plant generation. Others form sporulation bodies known either as ergots or chokes. Ergot- or choke-forming fungi may inhibit the seed set of the host grass (11). Otherwise, these fungi are not at all plant pathogenic. On the contrary, host grasses seem to derive several competitive advantages.

Herbivore resistance is only one of the benefits to plants hosting endophytic fungi. *A. lolii*-containing *L. perenne* had significantly increased shoot and root growth (12). *A. coenophialum*-containing *Festuca arundinacea* grown under controlled conditions had increased rate of photosynthesis, fresh weight gain, tiller production during regrowth, and decreased leaf roll during drought, all compared to genotypically identical, endophyte-free clones grown under equivalent conditions (3). In a 7-year field trial, endophyte-containing *F. arundinacea* was more resistant to crabgrass invasion and recovered quicker from summer drought stress than identical endophyte-free genotypes (13). The reasons for these selective advantages are not clear. In the *A. lolii/L. perenne* complex that produces lolitrem alkaloids, indole intermediates may mimic plant growth hormones, some of which are indole-derivatives. It is clear, however, that the alkaloids are responsible for the anti-herbivory effects.

**Peramine.** The lipophilicity of the pyrrolopyrazine nucleus in peramine, [3-(1,2-dihydro-2-methyl-1-oxopyrrolo[1, 2-a]pyrazin-3-yl)propyl]guanidine, (**1**) in Figure 1, is offset by the strongly basic guanidino substituent on the propyl side chain causing the molecule to be ionized at physiological pH. The two fused rings constitute a resonating system rendering this part of the molecule very stable.

*L. perenne* seeds contain at least 1 ppm of peramine (14). The concentrations in aereal parts of *L. perenne* containing *A. lolii* may be up to 40 ppm (dry weight) (15).

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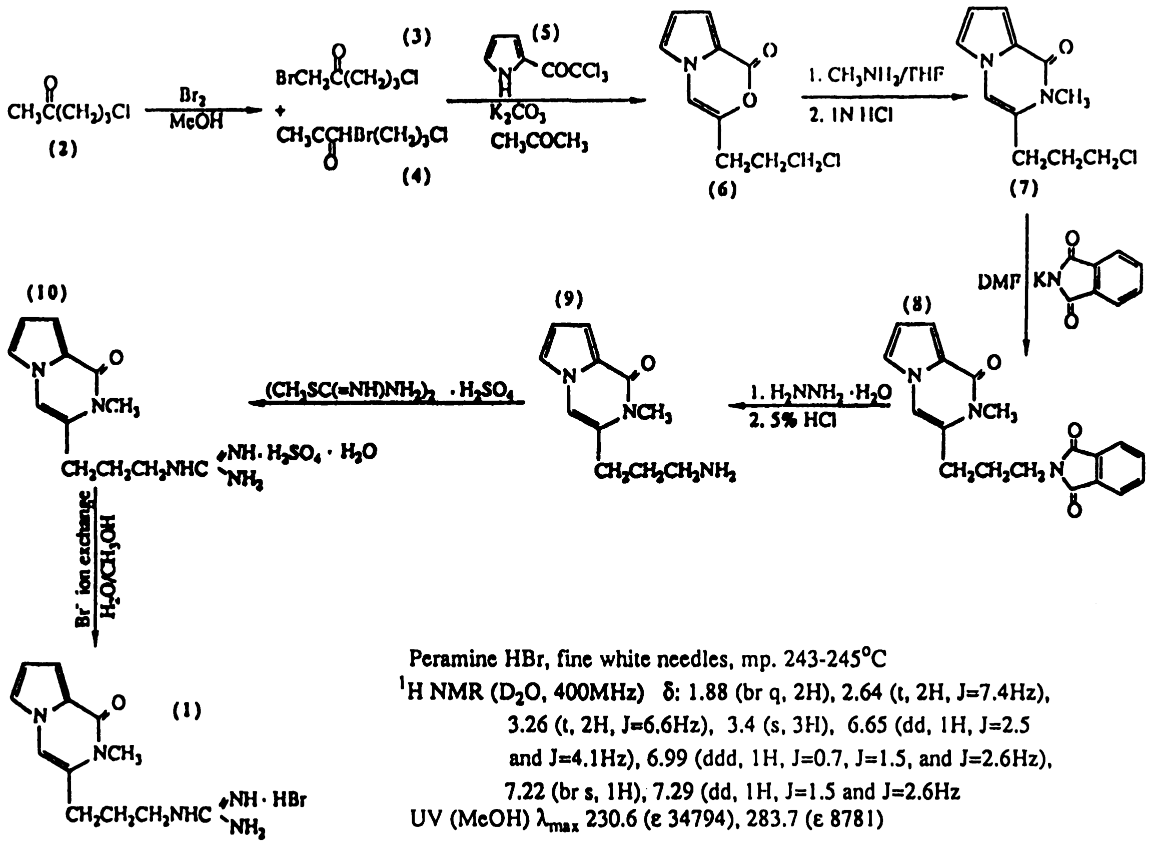
(2)
Br2
MeOH
(3)
(4)
(5)
K2CO3
CH3COCH3
(6)
1. CH3NH2/THF
2. 1N HCl
(7)
DMF
(8)
1. H2NNH2·H2O
2. 5% HCl
(9)
(CH3SC(=NH)NH2)2 ·H2SO4
(10)
Br⁻ ion exchange
H2O/CH3OH
(1)

Peramine HBr, fine white needles, mp. 243-245°C
$^1$H NMR ($D_2O$, 400MHz) δ: 1.88 (br q, 2H), 2.64 (t, 2H, J=7.4Hz), 3.26 (t, 2H, J=6.6Hz), 3.4 (s, 3H), 6.65 (dd, 1H, J=2.5 and J=4.1Hz), 6.99 (ddd, 1H, J=0.7, J=1.5, and J=2.6Hz), 7.22 (br s, 1H), 7.29 (dd, 1H, J=1.5 and J=2.6Hz
UV (MeOH) $\lambda_{max}$ 230.6 (ε 34794), 283.7 (ε 8781)

Figure 1. Scheme of peramine synthesis.

In Molecular Mechanisms of Insecticide Resistance; Mullin, C., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1992.

The total synthesis of peramine has been published (16-18). It is an antifeedant to a few insect species, notably the Argentine stem weevil (Listronotus bonariensis), and the intact pyrrolopyrazine nucleus is important for the feeding deterrent properties of the molecule (19).

**Lolitrem B.** This indole alkaloid, a large, highly complex and lipophilic diterpene (Figure 2) is neurotoxic, causing tremors and incoordination in mice and is toxic to L. bonariensis larvae (8, 20-22). Because of the complexity of the molecule, no structure/activity studies have been performed. The interactions with target or detoxifying macromolecules are unknown.

The content of lolitrem B in L. perenne leaves and stems is between 1 and 5 ppm, and the highest content is in the leaf sheaths; the levels increase with the age of plants and can reach 25 ppm in leaf sheaths of 5-week old grass (23).

**Effects of Endophyte-Containing Grasses and Alkaloids on Insects.** L. bonariensis shows a feeding and oviposition preference for endophyte-free L. perenne when offered a choice. This effect is related to the antifeedant properties of peramine and the toxicity of lolitrem B (9, 24). Pure peramine is not toxic to L. bonariensis larvae (19). Endophyte-containing L. perenne also deters feeding by one aphid species (Rhopalosiphum maidis) out of four (25) and a bluegrass billbug (26). Significantly reduced feeding and oviposition by several sod webworm species were also seen on endophyte-containing L. perenne compared to the endophyte-free clone (27, 28).

House crickets fed endophyte-containing L. perenne suffered 100% mortality within 4 days compared to 20-40% of those fed endophyte-free grass. The epithelium of the crop and proventriculus was destroyed in dying crickets (29), apparently causing septicemia. The effect looks similar to the gross toxic effect seen with several other, unrelated toxicants, e.g., tannins (30), cyanide (31), or the δ-endotoxin of Bacillus thuringiensis (32), implying interaction with a protein in the gut epithelium. Endophyte-containing L. perenne is also lethal to third and fourth instar S. eridania larvae (33).

Endophyte-containing L. perenne slows down the growth and development of Spodoptera frugiperda caterpillars but has only slight toxic effects (34-36), implying evolving defense mechanisms in this notoriously grass-feeding species. Significant differences in S. frugiperda larval body weight and food consumption were also seen when larvae fed on an endophyte-containing clone of L. perenne with a high concentration of A. lolii mycelia (37).

**Cytochrome P450.** Microsomal cytochrome P450 is of major importance for the metabolism of xenobiotics in all aerobic organisms, helping herbivorous insects to adapt quickly to new food sources that contain potentially toxic

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allelochemicals. The versatility of this enzyme resides mainly in its occurrence in multiple isoenzymic forms, its ability to be induced, and its ability to oxidize many different substrate molecules; the system is also inhibited by several types of allelochemicals. There is a vast literature about this enzyme system; see 38-41 for recent comprehensive reviews about insect cytochrome P450.

Each organism, perhaps even each tissue, has a set of several cytochrome P450 isozymes, some of which accept many different types of molecules as substrates and others of which may be highly substrate specific. In most cases, each cytochrome P450 is coded for by its own gene; 154 distinct P450 genes are assigned to 27 families (42, 43). Ten of them occur in all mammals studied to date and are organized into 18 subfamilies each consisting of a cluster of tightly linked genes. The expression of these genes is regulated by endogenous factors such as developmental stage, cell-type specific signals, and hormones, and by external factors such as inducing chemicals. Most of the P450 molecular genetics has been done with small laboratory mammals, but recent studies indicate that a multiplicity similar to that in mammals also occurs in insects (45, 46). The molecular genetics of cytochrome P450 is reviewed in several recent papers (42, 43, 46-48).

Several insect cytochrome P450 isozymes associated with resistance to insecticides or toxic plant allelochemicals have been characterized. A cytochrome that detoxifies pyrethroid insecticides was isolated from a strain of *Musca domestica* highly resistant to pyrethroids (49). Two phenobarbital-induced cytochromes were isolated from a strain of *M. domestica* with high resistance to organophosphate insecticides (50). Two isozymes were isolated from *Drosophila melanogaster* and one was associated with resistance to phenylurea (51). *Papilio polyxenes* caterpillars have a P450 that is induced specifically by furanocoumarins and detoxifies these compounds (52, 53).

Cytochrome P450 can be induced by a large number of compounds (47, 54). Induction can result in more of the original activities if a major, non-specific isozyme is induced or in a new activity if a specific, originally minor isozyme is induced. There is some specificity in the inducer action. It was clear early that polycyclic aromatic hydrocarbons (PAHs) induce a form of cytochrome P450 that oxidizes PAHs and not many other types of compounds. Phenobarbital, on the other hand, induces a broad spectrum of activities, including more of the original activities.

## Interactions between pure peramine and cytochrome P450

**Peramine Synthesis.** Peramine was synthesized as described (18) with minor modifications (Figure 1). Peramine bromide was used throughout our experiments. Control experiments included an equimolar dose of potassium bromide and showed that there were no effects from the bromide ion.

**Gross Effects of Peramine on S. eridania.** Unlike whole endophyte-containing L. perenne, pure peramine is not acutely toxic to S. eridania larvae and doesn't inhibit feeding or growth in this species. When a diet with 0.1% pure peramine was fed ad libitum to either third instar larvae until pupation or to last instar larvae, development rates were not different from those of the controls (Table 1). It should be noted that 0.1% is a very high concentration of peramine; L. bonariensis stops feeding on diets containing 1 ppm (55). The data in Table 1 show that S. eridania is a good model insect for studying the molecular effects of peramine because the experimental insects are neither sick nor starving.

**Table 1. Effects of dietary peramine on 6th instar S. eridania larvae**

| Fitness Factor | Control | Peramine |
|---|---|---|
| Mortality | 0 | 0 |
| Feeding rate (g/larva, 3 days) | 5.9 (0.5) | 5.9 (0.5) |
| Average weight of larva after 3 days (mg) | 732 (75) | 796 (81) |
| Average weight of pupae (mg) | 191 (29) | 225 (27) |

Groups of 30 newly molted 6th instar larvae were fed either control or 0.1% peramine diet (57) (in 3 days, each larva ingested 5.6 mg peramine). There are no differences between the means (Student's T-test). In another experiment (data not shown), 3rd instar larvae were started on the same kinds of diets and reared to pupation, also showing no differences in growth or feeding rates. Numbers in parenthesis are S.E. (N=30).

**Molecular Effects of Peramine.** Preliminary studies of peramine metabolism in vitro indicate that peramine is metabolized only slightly by cytochrome P450 in tissue fractions from midguts of S. eridania or S. frugiperda or livers from mice, sheep, or cattle (56). About 85-90% is excreted unmetabolized within 36 hrs in last instar S. eridania caterpillars (56). This is consistent with the resonance stability of the fused ring system and the ionization at physiological pH of peramine. When S. eridania last instar caterpillars had fed on a 0.1% peramine-containing diet for three days, there was insignificant inhibition of aldrin epoxidation. However, N-demethylation of p-chloro N-methylaniline was 52% of control activity, and O-demethylation of methoxyresorufin was 32% of control

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**Table 2. Effects of dietary peramine on activities of midgut defensive enzymes in S. eridania 6th instar larvae**

| Activity | Control | Peramine |
|---|---|---|
| Cytochrome P-450 (nmol/mg protein) | 0.36 (0.07) | 0.48 (0.04) |
| N-Demethylation (nmol/min, mg protein) | 4.22 (0.5) | 2.38 (0.5) |
| O-Demethylation (pmol/min, mg protein) | 18.10 (1.9) | 7.0 (0.8) |
| Epoxidation (nmol/min, mg protein) | 2.98 (0.3) | 2.46 (0.3) |
| Microsomal Esterases (umol/min, mg protein) | 14.50 (1.5) | 15.66 (1.6) |
| Soluble Esterases (umol/min, mg protein) | 56.01 (5.5) | 62.01 (6.5) |
| Glutathione Transferase (umol/min, mg protein) | 0.75 (0.08) | 0.72 (0.08) |

Groups of 30 larvae were fed a control diet or a diet containing 0.1% peramine for 3 days beginning immediately after molting to the 6th instar. Midgut microsomes and soluble fractions were used for the assays (62). Total P450 content was estimated with the carbonyl ferrocytochrome difference spectrum (63). The substrate for N-demethylation was p-chloro N-methylaniline; for O-demethylation, methoxyresorufin; for epoxidation, aldrin; for esterases, 1-naphthylacetate; for glutathione transferase, 1-chloro-2,4-dinitrobenzene (57, 64, 65). The data in parenthesis are S.E. (N=3). Only the differences in the N- and O-demethylation data are significant (Student's T-test).

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activity (Table 2). Peramine has no inductive or inhibitory effect on general esterase or glutathione transferase activities (Table 2). The microsomal carbonyl ferrocytochrome P450 difference spectrum was consistently 133% of control; this is not a statistically significant difference, whether or not it is biologically significant, depends on the P450 isozyme(s) affected by peramine.

SDS-PAGE (Figure 3) shows an apparent small increase in a minor cytochrome P450 band but no apparent decrease in any of the other cytochrome P450 bands; this is difficult to quantify without a purified cytochrome P450 fraction.

After feeding sixth instar S. eridania larvae on a diet containing 0.1% peramine for three days, the $LD_{50}$ of carbaryl was half of that to insects fed a control diet; carbaryl is detoxified exclusively by P450 (61). There were no differences in the toxicities of fluvalinate, a pyrethroid that can be detoxified by esterase hydrolysis

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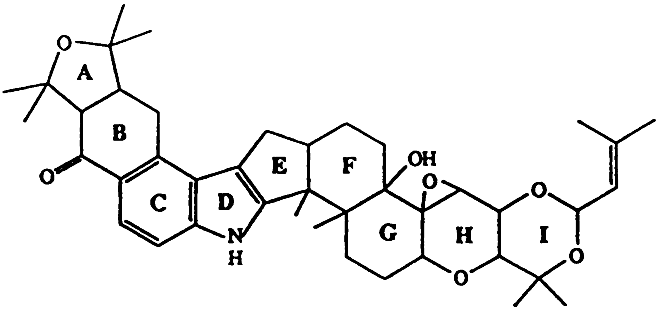

Figure 2. Structure of lolitrem B.

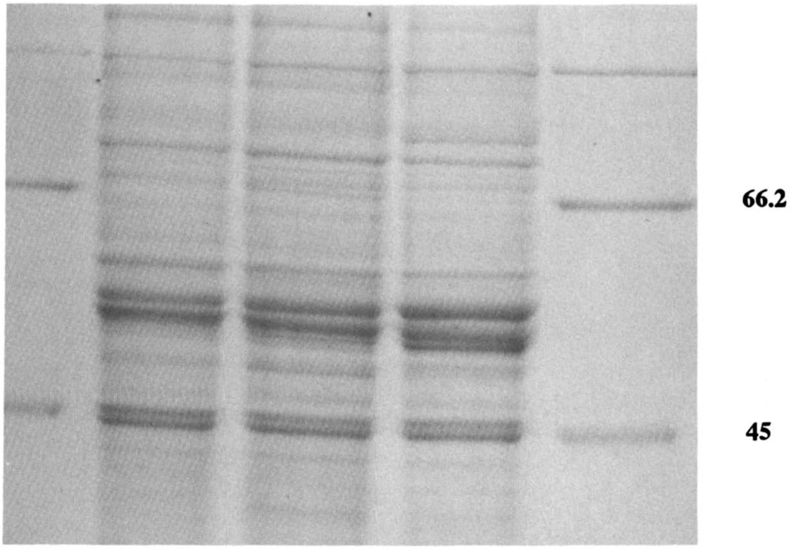

Figure 3. SDS-PAGE of microsomal protein from S. eridania midguts. The protein was from larvae that had fed on a control diet (left lane), a diet containing 0.1% peramine (middle lane), and a diet containing 0.2% pentamethylbenzene, an inducer of insect cytochrome P450 (58). Seventy ug of protein from washed microsomes in 10 mM Tris with mM EDTA, pH 7.5, were added to each lane. The running gel was 8%, and the gel was run at a constant current of 20 mAmps for the stacking gel and 30 mAmps for the running gel, modified from (59). The gel was stained with purified Coomassie brilliant blue G-250 according to (60). The molecular weight standards (in the outside lanes) were from Biorad (161-0304). The putative P450 bands show estimated molecular masses of 56, 53.8 (large bands), 53 (increased by pentamethylbenzene and peramine), and 52.7 kD (small bands).

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as well as by P450-catalyzed oxidation, or chlorpyrifos, an organophosphate that can also be detoxified by esterases or glutathione transferases (Table 3). The toxicities of the three insecticides widely used on turf are, thus, consistent with the pattern of effects on the insecticide-detoxifying enzymes (Table 2).

**Table 3. Toxicity of turf insecticides to 6th instar *S. eridania* larvae**

| Insecticide | Control Diet | | 0.1% Peramine Diet | |
|---|---|---|---|---|
| | $LD_{50}$ | $LD_{95}$ | $LD_{50}$ | $LD_{95}$ |
| Carbaryl | 265 | 420 | 130 | 230 |
| Fluvalinate | 4.2 | 10 | 4.2 | 10 |
| Chlorpyrifos | 1.6 | 3.7 | 1.5 | 3.7 |

The toxicities were measured 24 hours after topical application to groups of 10 larvae. Five insecticide concentrations were used, each repeated three times. After treatment the larvae were held at 22°C and provided with control diet.

**Conclusions.** Endophytes have a great potential for safe and selective insect control in areas where turfgrasses are used for home or urban landscaping. Endophytes may also, in the future, be engineered into crop plants and combined with a minimized spray application of synthetic insecticides. There are certain strains of *A. lolii* that biosynthesize peramine but not the lolitrems (Popay, 1990 personal communication) that may be used in conjunction with synthetic insecticides. Variations and permutations in the combinations of endophyte alkaloids and insecticides will help reduce the evolution of resistance in insects.

Our research shows interactions of peramine with cytochrome P450. The inhibitory effects imply that peramine could be a synergist at least for carbamate insecticides. Peramine may also be a synergist for co-occurring, toxic lolitrems or ergot alkaloids. This would not be a unique case; many plants in addition to *Chrysanthemum cinerariefolium*, "the pyrethrum flower" contain lignans (compounds that inhibit cytochrome P450 by a benzodioxole group) and other synergists together with one or more toxicants, e.g., the parsnip (66) and Piperaceae peppers (67).

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# Chapter 11

# Resistance to Plant Allelochemicals in *Heliothis virescens* (Fabricius)

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Potential metabolic routes for the resistance of tobacco budworm (TBW) larvae to host plant allelochemicals nicotine, 2-tridecanone and quercetin were explored. Midgut preparations from larvae resistant to nicotine and 2-tridecanone had elevated levels of cytochrome P450 which were associated with significant increases in metabolism for five of six monooxygenase substrates. In quercetin tolerant larvae, metabolism of two monooxygenase substrates was significantly enhanced although no increase in P450 content was observed. Glutathione transferases and esterases did not appear to be involved in the resistance of any of the strains examined. Patterns of substrate oxidations varied between strains and inducing agents, suggesting that different isozymes of P450 are associated with resistance and induction.

The tobacco budworm (TBW), *Heliothis virescens* (Fabricius), is a polyphagous insect which has been observed feeding on 31 different plant species in as many as 14 plant families (*1*). These various host plants produce fitness reducing and/or antifeedant chemicals as defenses (*2*). Some of the toxic allelochemicals encountered by the TBW in its natural host plants include gossypol and related terpenoids, condensed tannins, quercetin, rutin, anthocyanin, nicotine and flavonoids (*3*).

## Mechanisms for Tolerance to Allelochemicals and Pesticides

At least three different mechanisms for dealing with host plant allelochemicals have been demonstrated in TBW. Nicotine tolerance was postulated to be the result of an efficient excretory system, like that previously demonstrated in the tobacco hornworm (*4*). This idea was supported by the lack of discernible

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metabolites in TBW larvae and their feces ten hours after treatment with 200 µg free base nicotine (*5*). Behavioral adaptations have also been observed in TBW. Young larvae feeding in cotton avoid gossypol-producing glands (*6*). As they become older and less susceptible to gossypol (*7, 8*), presumably as a result of allelochemically-based induction of detoxifying enzymes (*9, 10*), non-selective consumption of gossypol-containing glands occurs (*11*). Recent studies suggest that the tobacco budworm utilizes its inducible metabolic system for the detoxication of a variety of allelochemicals (*9, 10, 12, 13*).

Studies of enzyme induction by host plants and/or allelochemicals derived from host plants demonstrate that induced insects can detoxify pesticides faster than non-induced insects (see ref. *14* for review). For example, sixth instar larvae of the variegated cutworm, *Peridroma saucia* (Hubner), had greater tolerance to acephate, methomyl and malathion when reared on peppermint leaves versus bean leaves. The increase in tolerance was associated with a significant increase in monooxygenase activity (*15*). Host plant induction of enzyme systems other than monooxygenases, such as glutathione S-transferases, are also associated with insecticide tolerance (*16, 17*). Results of these studies suggest that changes in the chemistry of the host plants consumed by a particular insect species (e.g. after the introduction of a resistant host genotype) may influence the susceptibility of the consumer to insecticides. Hence, tolerance of host plant allelochemicals and insecticides are related.

Relatively few insects with intraspecific genetic variation with respect to allelochemically-based resistance are in culture. Yet, an understanding of such genetically based resistance is necessary in order to predict which pesticides might best be utilized in combination with host plant resistance. In addition, little information is available concerning mechanisms of genetic resistance to allelochemicals. After over 30 generations of laboratory selection, several strains of tobacco budworm have been developed with resistance to allelochemicals (*18, Gould, unpublished data*). For our studies, we selected strains possessing heritable resistance to nicotine (WAKE-N), 2-tridecanone (WAKE-T), and quercetin (BC-Q). The control strains from which the resistant strains were selected included NC-1 (from which BC-Q was selected) and WAKE (from which WAKE-T and WAKE-N were selected).

These studies utilized midgut homogenates from larvae reared on diets in the absence of the selecting agent for the preparation of microsomes (100,000 g pellet) and cytosolic (100,000 g supernatants) fractions. Substrates utilized for esterase and glutathione transferase enzymes included α-naphthyl acetate and 1-chloro, 2,4-dintrobenzene, respectively. For monooxygenase determinations, several substrates were selected in an effort to represent a variety of metabolic possibilities. These included p-nitroanisole and methoxyresorufin (*O*-demethylation), benzphetamine (*N*-demethylation), benzo(a)pyrene (aryl hydroxylation), lauric acid (alkyl hydroxylation), and phorate (sulfoxidation).

## Resistance Development to Pesticides vs Allelochemicals

Prior to the introduction of the pyrethroids, the tobacco budworm had developed resistance to nearly every insecticide used against it, including DDT, carbaryl,

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endrin, parathion, EPN, and monocrotophos (*19*). Tobacco budworm resistance to pyrethroids was first diagnosed in populations collected from western Texas where field control failures had occurred (*20*). Since that time, pyrethroid resistance has been documented in several southeastern states (*20-23*) however, pyrethroid management plans adopted in these areas have likely curtailed the development of extremely high resistance levels (*24*). The likelihood that pyrethroid resistance would eventually develop in TBW was suggested by a laboratory study in which selection pressure was exerted at a level near 80% mortality. At this rate, significant levels of resistance (37-fold) were attained within 11 generations (*25*). By 36 generations, this strain had attained resistance levels of > 1000-fold (*26*). Although pyrethroid resistance in field populations have not yet approached either of these levels, with intense population pressure the possibility for such dramatic increases does exist (*27*).

Contrasting with the high resistance levels attained by selection with synthetic insecticides, selection of TBW larvae with various allelochemicals has not resulted in high levels of resistance. After more than 30 generations of selection pressure for nicotine and 2-tridecanone resistance using dietary concentrations resulting in acute toxicity for up to 80% of the population, resistance levels at the $LC_{50}$ do not exceed 2.5-fold (*Gould, unpublished data*).

Selection for tolerance to quercetin was based upon the ability to grow on quercetin containing diets, rather than on mortality. Concentrations of 0.5% quercetin were non-lethal, but severely limited growth of non-adapted larvae (Table I). Larvae selected for tolerance to quercetin, however, attain normal body weight within the same time period as larvae reared in absence of 0.5% quercetin.

**Resistance to 2-Tridecanone.** Dimock and Kennedy (*28*) demonstrated that first instar cotton bollworm, *H. zea*, placed on leaves of accession PI 134417 of the wild tomato, *Lycopersicon hirsutum f glabratum*, C.H. Mull, were quickly paralyzed, even in absence of foliar consumption. This paralytic response was postulated to be due to the fumigant action of 2-tridecanone emanating from glandular trichomes of the tomato leaf. The quick recovery of exposed larvae suggested involvement of an inducible detoxication system. Exposure of neonates (*29*) and/or eggs (*30*) to 2-tridecanone resulted in greater tolerance to subsequent exposures of both 2-tridecanone and the insecticide carbaryl.

**Table I. Effects of Quercetin on the Larval Growth of Tobacco Budworm**[a]

| | Body Weight (mg)[b] | |
|---|---|---|
| *Diet* | *Control* | *BC-Q* |
| Regular | 252.6 ± 62.9 | 222.8 ± 71.3 |
| + Quercetin | 17.4 ± 5.3* | 232.6 ± 75.2** |

[a] Body weights taken 10 days following inoculation of first instar larvae.
[b] Mean ± standard deviation for 20 larvae.
*p<0.01 vs. regular diet; **p<0.01 vs control strain.

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Fifth instar TBW larvae fed diets of 2-tridecanone or tomato leaves had significant increases (2.2 - 3.1-fold) in gut cytochrome P450 and glutathione transferase activities (*31*). The increases in P450 content were accompanied by qualitative differences in binding spectra often associated with insecticide resistance. These differences included an increased Type I difference spectrum, a shift in the λ-max of the CO-difference spectrum from 450 to 448 nm, and an increase in the magnitude of the Type II spectrum (*32*). In addition, larvae fed on diets containing 2-tridecanone were more tolerant of diazinon and more able to degrade diazinon and its oxon than untreated larvae (*31*).

In our study of larvae possessing genetic resistance to 2-tridecanone, midgut cytochrome P450 levels were two-fold greater than in susceptible larvae (*13*). Measurements of glutathione transferase (1-chloro 2,4 dinitrobenzene (CDNB)) and esterase activities (α-naphthyl acetate (α-NA) and *p*-nitrophenol acetate (PMPA)), did not indicate statistical differences between 2-tridecanone susceptible (WAKE) and resistant (WAKE-T) strains (Table II). Three of six monooxygenase substrates surveyed showed significant increases in metabolism (benzo(a)pyrene (2.6-fold), benzphetamine (2.1-fold) and phorate (1.8-fold) when activity is expressed per mg of microsomal protein (*13*)). Standardization of the data with respect to P450 content, however, (Table III) indicated that the increases associated with 2-tridecanone resistance resulted from a general increase in P450 content rather than from an isozyme-specific increase.

**Resistance to Nicotine.** The tobacco budworm is one of several insects which successfully feeds on nicotine-containing tobacco. The green peach aphid, ***Myzus persicae*** (Sulz.), avoids nicotine by selectively feeding in the phloem (*33*), while

**Table II. Cytochrome P450, Esterase and Glutathione Transferase Activities in Tobacco Budworm Strains**

| *Strain*[a] | *P450*[b] | *α-NA*[c] | *PNPA*[c] | *CDNB*[c] |
|---|---|---|---|---|
| NC-1 | 0.25 ± .03 | 175 ± 80 | 33 ± 12 | 228 ± 69 |
| BC-Q | 0.35 ± .04 | 142 ± 31 | 42 ± 14 | 222 ± 53 |
| WAKE | 0.30 ± .05 | 150 ± 68 | 35 ± 11 | 320 ± 142 |
| WAKE-T | 0.60 ± .11 | 111 ± 34 | 20 ± 18 | 248 ± 137 |
| WAKE-N | 0.62 ± .05 | 214 ± 36 | 27 ± 17 | 236 ± 109 |

[a] The quercetin resistant (BC-Q) strain was derived from the susceptible (NC-1) strain, while the 2-tridecanone (WAKE-T) and nicotine (WAKE-N) resistant strains were derived from the susceptible (WAKE) strain.

[b] P450 expressed as nmoles/mg protein ± SE.

[c] Esterase (1-naphthyl acetate (α-NA) and p-nitrophenyl acetate (PNPA)) and glutathione transferase (1-chloro, 2,4-dinitrobenzene (CDNB)) activities expressed as nmole/min/mg protein ± SD.

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**Table III. Monooxygenase Activities[a] for Various Substrates[b] in Resistant and Susceptible Strains[c] of the Tobacco Budworm**

| *Strains* | *PNA* | *Benz* | *B(a)P* | *MRR* | *Phorate* | *LA* |
|---|---|---|---|---|---|---|
| NC-1 | 2.8 | 6.4 | 0.44 | 0.05 | 8.4 | 10.5 |
| BC-Q | 4.1 | 6.4 | 0.71 | 0.03 | 12.1 | 10.1 |
| WAKE | 4.6 | 7.2 | 0.30 | 0.05 | 24.7 | 13.3 |
| WAKE-T | 3.4 | 7.6 | 0.38 | 0.04 | 22.5 | 7.8 |
| WAKE-N | 5.6 | 12.8 | 0.66 | 0.06 | 18.6 | 8.9 |

[a] Adapted from ref. *13*.

[b] Substrates are *p*-nitroanisole (PNA), benzphetamine (Benz), (B(a)P), methoxyresorufin (MRR), phorate and lauric acid (LA). Means are expressed as nmole/min/nmole P450, with exception of MRR and B(a)P are expressed as fluorescence units/min/nmole P450.

[c] For description of strains, see Table II.

the tobacco hornworm, *Manduca sexta* (L), the TBW and the cabbage looper, *Trichoplusia ni*, (Hubner), efficiently utilize excretory mechanisms to eliminate foliar nicotine in their diet (*4, 5*). In other tobacco feeding insects including the tobacco wireworm, the cigarette beetle and the grasshopper, nicotine was readily metabolized via hydroxylation followed by alcohol dehydrogenation to form the nontoxic metabolite, cotinine (*5*). In the tobacco hornworm, rapid elimination of nicotine appears to be the primary resistance mechanism, however, nicotine has also been shown to be metabolized by the central nervous system (*34*).

While nicotine metabolism has not yet been examined in the nicotine resistant TBW strain, several lines of evidence suggest involvement of enhanced metabolic detoxication in comparison to the susceptible strain. Resistant larvae can tolerate two fold more dietary nicotine than their susceptible counterparts. These larvae had an increase in cytochrome P450 content (Table II) and significant increases in monooxygenase activities towards benzo(a)pyrene (4.6-fold), benzphetamine (3.7-fold) and phorate (1.5-fold) relative to the susceptible WAKE strain (*13*). Esterase and glutathione transferases do not seem to be involved in nicotine resistance in TBW (Table II). In contrast with the 2-tridecanone selected strain, the nicotine resistant budworm had some isozyme specificity for the substrates benzo(a)pyrene and benzphetamine as indicated by P450 based activities (Table III). Since hydroxylation and N-demethylation are predominant metabolic pathways for nicotine oxidation in mammals (*35*), the specificity of P450 in nicotine resistant TBW for benzo(a)pyrene and benzphetamine may suggest enhanced cotinine and nornicotine production, respectively. Metabolism of nicotine to cotinine by some insects has been reported, although not in the tobacco budworm (*5*). Further investigations to identify metabolites and their rate of formation in these strains should resolve these questions.

**Resistance to Quercetin.** The incorporation of quercetin (0.05%) into TBW diets resulted in 50% inhibition of larval growth (*36*). The mechanism by which quercetin inhibits TBW growth is not known, however, it is not the result of repellancy, since larvae show a slight preference for quercetin-containing diets (*Gould, unpublished data*).

There is some evidence suggesting that quercetin exerts its toxic action in Lepidopterous larvae by means of oxygen toxicity (*37*). Upon ingestion, quercetin is activated by one electron oxidation to a free radical o-semiquinone, which reacts with $O_2$ to generate the superoxide anion radical. This oxygen radical subsequently forms hydrogen peroxide and hydroxy radicals. These reactive products cause cell damage through lipid peroxidation and enzyme inactivation (*38*). Mechanisms of resistance to prooxidants include detoxication by cytochrome P450 (*39, 40*) as well as increased levels of several antioxidant enzymes such as superoxide dismutase (SOD), catalase, and quinone reductase (*37, 41*).

The quercetin tolerant TBW strain did not differ in P450 content from the susceptible strain it was derived from (Table II). However, increases in benzo(a)pyrene hydroxylation (2.5-fold) and phorate sulfoxidation (2-fold) were found to be associated with quercetin tolerance (*13*). Normalization of the data with respect to P450 content suggests that benzo(a)pyrene hydroxylation is isozyme specific (Table III). This suggests the possibility of monooxygenase involvement in quercetin tolerance, however, its level appears to be small.

Several lines of evidence suggest that quercetin toxicity can be overcome by increases in prooxidant enzymes such as SOD. 1. Insect species known to feed on plants containing high levels of prooxidant allelochemicals tend to have higher levels of antioxidant enzymes (*41, 42*). Dietary exposure of these insects to prooxidant compounds results in rapid increases in SOD activity (*37, 41, 42*). 3. The application of an inhibitor of SOD (diethyldithiocarbamate) to larvae feeding on quercetin-containing diets resulted in a significant decrease in the relative growth rate (*42*).

In our preliminary studies, using the SOD method described by McCord and Fridovich (*43*), quercetin tolerant larvae had slightly greater SOD activities than did the susceptible strain (SOD levels (mean ± S.D. of two replicates) in quercetin tolerant and susceptible larvae were 4.48 ± 0.32 and 3.53 ± 0.05 Units/mg protein, respectively). The increase in SOD activity observed in the quercetin tolerant strain, though small (1.3-fold), suggests that SOD may be a component of the tolerance of this strain to quercetin. Further characterization of this and other associated enzyme systems are being explored.

## Pesticide Interactions with Allelochemical Resistance

Host plant resistance is one of the more promising components of integrated pest management (*44*). However, interactions between host plant constituents and enzyme induction in insects raise questions with respect to the use of insect-resistant genotypes and their potential influences upon insecticide susceptibility. An insect population feeding upon a resistant host may experience reduced vigor

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as a result of nutritional deficiency, nonpreference for feeding, or antibiosis; all of which would tend to contribute toward increases in insecticide susceptibility. Indeed, there are several examples in which host plant resistance has contributed to increased pesticide susceptibility (*45-47*). However, insects surviving on a resistant host plant may have an increased capacity for detoxication which would result in decreased insecticide susceptibility.

In our studies, TBW larvae possessing resistance to dietary quercetin were significantly cross resistant to methyl parathion and its activation product, methyl paraoxon; as well as to methomyl and fenvalerate (Table IV). No strain differences in methyl parathion penetration (Figure 1) or in its inhibition of acetylcholinesterase activity (Table V) accounted for this cross resistance.

Application of piperonyl butoxide to both strains 4 hours prior to insecticide application resulted in significant antagonism for both methyl parathion and methyl paraoxon toxicity, suggesting that activation of parathion to paraoxon and subsequent detoxication of the methyl paraoxon by monooxygenases was significantly inhibited (Table VI). Pretreatment with an esterase inhibitor, DEF, synergized methyl parathion toxicity to the quercetin tolerant strain but had no effect on the control strain. A similar effect of DEF was also was observed with respect to paraoxon toxicity in both strains. Addition of dietary quercetin prior

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**Table IV. Toxicity of Insecticides to 5th Instar Tobacco Budworm Larvae**

| | *$LD_{50}$ Value (µg/g)* | | *Resistance* |
|---|---|---|---|
| *Insecticide* | *NC-1* | *BC-Q* | *Ratio*[a] |
| Methyl Parathion | 11.3 | 28.6 | 2.5 |
| Methyl Paraoxon | 5.5 | 14.4 | 2.6 |
| Methomyl | 1.5 | 3.3 | 2.2 |
| Fenvalerate | 0.4 | 1.2 | 2.6 |

[a] Resistance ratio = $LD_{50}$ for BC-Q strain / $LD_{50}$ for NC-1 strain.

**Table V. Inhibitory Activity of Methyl Paraoxon on Acetylcholinesterase Activity of Tobacco Budworm**

| *Strain* | *$I_{50}$ Value (1 x $10^{-5}$M)*[a] |
|---|---|
| NC-1 | 4.5 ± 0.9 |
| BC-Q | 3.6 ± 0.6 |

[a] Figures represent mean value ± standard deviation of three replicates.

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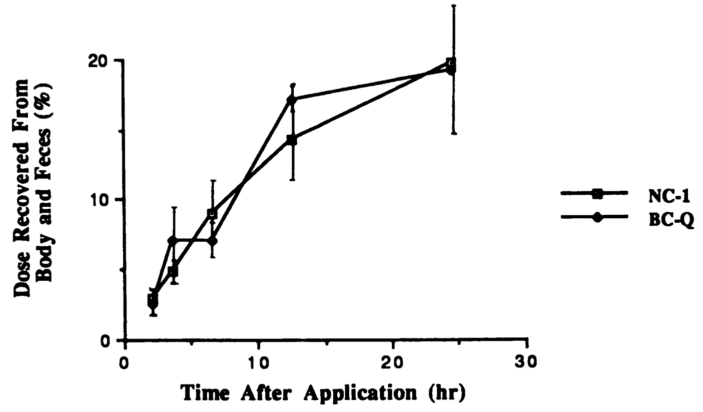

Figure 1. Penetration of Methyl Parathion in 5th Instar Tobacco Budworm Larvae. Data represents mean values ± standard deviations of three replicates.

Table VI. Effect of Synergists[a] on Organophosphate Toxicities to 5th Instar Tobacco Budworm Larvae[b]

| | *NC-1* | | *BC-Q* | |
|---|---|---|---|---|
| *Insecticide* | *LD$_{50}$*[c] *(μg/g)* | *Synergist Ratio*[d] | *LD$_{50}$* *(μg/g)* | *Synergist Ratio* |
| Methyl Parathion | 10.8 | 1.0 | 25.2 | 1.0 |
| +PB[e] | 103.0* | 0.1 | 197.0* | 0.1 |
| +DEF[f] | 11.8 | 0.9 | 17.4* | 1.5 |
| +Quercetin | 25.0* | 0.4 | 14.4* | 1.8 |
| Methyl Paraoxon | 5.0 | 1.0 | 12.1 | 1.0 |
| +PB | 12.0* | 0.4 | 13.5 | 0.9 |
| +DEF | 5.4 | 0.9 | 5.5* | 2.1 |
| +Quercetin | 8.2* | 0.6 | 5.8* | 2.1 |

[a] Each synergist except quercetin was applied topically at 100 μg/larvae 4 hours prior to insecticide application. Dietary quercetin was at 0.5%.

[b] Adapted from ref. *48*.

[c] *$p < 0.05$ vs LD$_{50}$ for the insecticide alone.

[d] Synergistic ratios (LD$_{50}$ for the insecticide alone/LD$_{50}$ for the insecticide and the synergist).

[e] Piperonyl butoxide.

[f] S,S,S,-tributyl phosphorothioate.

to insecticide treatment resulted in significant decreases in toxicity of both parathion analogs for the susceptible strain, while increasing toxicity of these compounds to the quercetin tolerant strain.

Further investigation into metabolism of methyl parathion revealed that the quercetin tolerant strain produced less of the activation product (methyl paraoxon) and had increased production of the p-nitrophenyl glucoside (Table VII). In a previous study, resistance of a methyl parathion resistant (55-fold) strain was attributed to differences in metabolism which were similar to those observed in the quercetin resistant strain. However, the methyl parathion resistant strain also had significant reductions in penetration of the pesticide (*49*). The disparity in resistance levels of these two strains is not readily explained on the basis of observed differences in penetration and metabolism, but may involve a combination of the two mechanisms.

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This is not the only example of cross resistance between host plant allelochemicals and pesticides. Spider mites selected for survival on a toxic host (cucumber) were more tolerant to two organophosphate insecticides than those reared on a susceptible host (*50*). These examples are also typified by many studies in which induction by host plants and/or allelochemicals has been demonstrated to result in decreased insecticide toxicities (*11, 15, 16, 31*).

For example, TBW reared on a resistant variety of tomato had significant increases in tolerance to carbaryl and diazinon, presumably due to the induction of monooxygenase enzymes by the allelochemical involved in the resistance mechanism of tomato (2-tridecanone) (*27, 29*). Similarly, addition of gossypol or lyophilized cotton flower buds to the diet of the tobacco bollworm induced increased tolerance to methyl parathion (*11*). Gossypol is also synergized by the monooxygnease inhibitor, piperonyl butoxide, however, it was not clear if such synergism was affected by behavioral factors such as non-preferential feeding (*8*). These results suggest that gossypol may, like 2-tridecanone, nicotine, and quercetin, induce monooxygenase enzymes required for its detoxication.

## Conclusion

Clearly, acquired resistance to host plant allelochemicals affects metabolic enzymes as well as an insect's susceptibity to insecticides. Understanding biochemical interactions produced by allelochemicals prior to their utilization in insect-resistant varieties of agronomic plants will help to avoid some of the pitfalls associated with synthetic insecticide use and ensuing resistances. A proper understanding of these interactions may actually enhance the probability that pesticides will augment host plant resistance.

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**Table VII. *In vivo* Metabolism of Methyl Parathion in 5th Instar Tobacco Budworm Larvae**

| *Minutes* |  | *Percentage of Dose Recovered* |  |  |  |  |  |  |
|---|---|---|---|---|---|---|---|---|
| *After Injection* | *Strain* | *Methyl Parathion* | *Methyl Paraoxon* | *p-Nitro-Phenol (pNP)* | *pNP-glucoside* | *pNP-sulfate* | *Unknown 1*[b] | *Unknown 2*[c] |
| 20 | BC-Q | 71.8 ± 2.8 | 7.5 ± 1.9 | 5.2 ± 0.2 | 9.9 ± 0.5* | 1.6 ± 0.6 | 0.0 | 4.0 ± 0.5 |
|  | NC-1 | 77.5 ± 3.5 | 6.6 ± 1.5 | 4.3 ± 0.5 | 6.5 ± 1.0 | 1.5 ± 0.8 | 0.3 ± 0.2 | 3.5 ± 1.0 |
| 60 | BC-Q | 52.9 ± 0.7* | 15.0 ± 0.9 | 7.5 ± 1.4 | 14.6 ± 1.8* | 4.6 ± 1.6 | 0.0 | 5.2 ± 0.6 |
|  | NC-1 | 34.6 ± 3.2 | 16.6 ± 3.6 | 6.3 ± 0.2 | 29.9 ± 4.3 | 7.4 ± 3.6 | 0.4 ± 0.3 | 4.9 ± 0.8 |
| 180 | BC-Q | 27.4 ± 2.2 | 16.9 ± 0.9* | 9.9 ± 1.0 | 34.2 ± 1.6* | 6.6 ± 1.2 | 0.0 | 5.0 ± 1.1 |
|  | NC-1 | 24.2 ± 3.6 | 7.3 ± 1.3 | 7.3 ± 1.3 | 48.7 ± 3.6 | 5.6 ± 0.7 | 0.6 ± 0.4 | 6.3 ± 0.6 |
| 360 | BC-Q | 13.6 ± 1.8 | 17.6 ± 4.0* | 14.6 ± 1.8 | 37.0 ± 4.9* | 9.4 ± 1.5 | 0.7 ± 0.5 | 7.1 ± 0.5 |
|  | NC-1 | 13.2 ± 4.0 | 4.4 ± 1.1 | 9.6 ± 3.3 | 55.3 ± 9.3 | 8.7 ± 2.3 | 0.8 ± 0.7 | 8.0 ± 2.1 |

[a] Mean values ± standard deviation of three replications; $*p < 0.05$ vs NC-1 strain.

[b] Rf was 0.0 in 4:1 benzene:ethyl acetate and 0.07 in 80:3:27 n-butanol:ammonia:water.

[c] Rf was 0.0 in 4:1 benzene:ethyl acetate and 0.73 in 80:3:27 n-butanol:ammonia:water.

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## Chapter 12

# Resistance in the Diamondback Moth to Pyrethroids and Benzoylphenylureas

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Larvae of the diamondback moth (DBM), *Plutella xylostella* (L.), an insect pest of cruciferous vegetables, have a very effective microsomal P450-dependent monooxygenase system. Enhanced detoxication mediated by this enzyme is closely related to the rapid development in DBM of resistance to pyrethroids, the synergist piperonyl butoxide, and to benzoylphenyl ureas. Resistant DBM exhibit much higher microsomal monooxygenase activities toward a number of model substrates than susceptible strains. Qualitative and/or quantitative differences in cytochrome P450s, cytochrome b5, and cytochrome c reductase of microsomes from susceptible and resistant DBM larvae have been observed. The possible involvement of other detoxifying enzymes is also discussed.

Cruciferous vegetables are economically important throughout the world. Before the 1980s, the diamondback moth (DBM), *Plutella xylostella* (L.), was a serious pest mainly in southeast Asia where crucifers are often grown around urban centers by small landholders and farmers tend to adopt intensive practices (including spraying large quantities of insecticide cocktails) in order to protect their important source of cash income (*1*). However, in the past decade, crucifer production in some regions of east Asia, the Americas and Oceania has been seriously threatened by the attack of DBM (*2*). Larvae of this moth feed on the foliage of cruciferous plants from the seedling stage to harvest, and greatly reduce the yield and quality of produce. In addition to a high reproductive potential and adaptations to adverse conditions (*3*),

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this insect has an extraordinary capacity to become resistant to all classes of chemical insecticides (*4*). Recently, resistance to even the microbial insecticide ***Bacillus thuringiensis*** has been observed in field populations of DBM (*5*).

Here we will review current understanding of DBM resistance to pyrethroids and benzoylphenyl ureas, with a special emphasis on detoxication resistance mechanisms. Hopefully, this understanding will help to formulate integrated management programs for this notorious yet "respected" insect pest.

## Pyrethroid Resistance

Despite the occurrence and reputed occurrence of resistance to chlorinated hydrocarbon, organophosphorus and carbamate insecticides (*1,2*), DBM had not appeared unmanageable until the mid-1970s when photostable pyrethroids were discovered and widely used.

**Occurrence.** Fenvalerate was first registered in 1976 for DBM control in Taiwan, and a recent unofficial report pointed out that by 1977 resistance was already detected in the field. DBM larvae collected in 1979 from Ban-chau near metropolitan Taipei had approximately 90-fold resistance to fenvalerate as compared with a relatively susceptible local strain (*6*). Populations collected later from the same site were about 3000-fold resistant to fenvalerate as compared with a susceptible strain from France (*7*). Similar levels of resistance to cypermethrin and deltamethrin were also detected, although permethrin resistance was consistently lower. Despite existing carbamate and organophosphorus resistance in DBM, pyrethroid resistance was not found in Japan up through 1983 (*8,9*). Formulations of fenvalerate mixed with some organophosphorus insecticides were introduced and widely used that year, and by 1984 up to 12,000-fold fenvalerate resistance was found in DBM collected from southern Japan (*10*). DBM resistance to fenvalerate (*11*) and permethrin (*12*) together with resistance to other groups of insecticides was confirmed in Florida and other parts of the U. S. in 1988/1989. In addition, pyrethroid resistance has been reported in Australia (Altmann, J. A., Queensland Agricultural College, personal communication, 1988), Central America (*13*), Thailand (*14*) and Malaysia (*15*).

**Resistance Mechanisms.** In contrast with the widespread occurrence of DBM resistance to insecticides, information on resistance mechanisms is very limited. This situation is expected to change soon with the involvement of several experienced laboratories in DBM studies.

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**Non-metabolic Mechanisms.** Based on the cross resistance between DDT and pyrethroids and the absence of DDT synergism by some metabolic blocking agents, we proposed that a non-metabolic DDT resistance mechanism might also play an important role in DBM resistance to pyrethroids (*16*). Later, Hama obtained electrophysiological evidence to prove a decreased sensitivity of the central nerve system to fenvalerate in a pyrethroid resistant strain of DBM (*17*). A reduced cuticular penetration of *s*-fenvalerate in fenvalerate resistant DBM as compared with a susceptible strain has recently been observed (*18*).

**Metabolic Mechanisms.** From synergist studies, we first suggested that enhanced degradation by microsomal P450-dependent monooxygenases was a major mechanism of pyrethroid resistance in DBM (*19*). A rather limited involvement of carboxylesterase in permethrin resistance was also observed. Selection of DBM larvae with a combination of fenvalerate and piperonyl butoxide (PB) resulted in the loss of the synergistic action of PB, although the DBM remained susceptible to the action of two other inhibitors of microsomal monooxygenases, MGK 264 and sulfoxide (*20*).

DBM resistance to the synergistic action of PB was probably also caused by an enhanced microsomal degradation of this synergist (*21*). While DBM resistance to fenvalerate was quite persistent, its resistance to fenvalerate plus PB appeared unstable, and after removal of selection pressure, the insect regained its susceptibility to the synergistic action of PB within 5 generations (*20*).

Using model substrates, we showed that pyrethroid resistant DBM larvae had higher microsomal P450-dependent monooxygenase activities (epoxidation and *O*-dealkylation) than susceptible ones (Table I), and that increased activities in general paralleled the resistance levels. The difference in aldrin epoxidation was small (up to 2-fold), while differences in *O*-dealkylation were much higher, varying from 3 to 18-fold (*21,22*). Despite monooxygenase association with resistance, we have been unable to detect *in vitro* oxidation of pyrethroids either by monitoring substrate disappearance with gas chromatography or by measuring substrate-dependent NADPH consumption. Varying the experimental conditions and addition of cofactors and protectants have not improved rates of pyrethroid oxidation.

A methyl parathion-selected MPA strain (resistance ratio 455), which had only 5-fold fenvalerate resistance, had microsomal P450-dependent monooxygenase activities (except ECOD) not significantly different from those of the susceptible FS strain (Table I). Conjugation mediated by glutathione transferase has been proposed as the major mechanism of DBM resistance to some organophosphorus insecticides, in addition to a reduction of

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Table I. Microsomal monooxygenase activities of diamondback moth larvae with different levels of fenvalerate resistance

| | | Specific activity[c,d] | | | |
|---|---|---|---|---|---|
| Strain[a] | RR[b] | MROD | EROD | ECOD | AE |
| FS | 1 | 26.0±1.3 | 5.9±1.8 | 0.29±0.03 | 84.1±5.2 |
| | | (1.0) | (1.0) | (1.0) | (1.0) |
| MPA | 5 | 33.8±3.7 | 6.3±1.9 | 1.02±0.17 | 85.2±4.6 |
| | | (1.3) | (1.1) | (3.5) | (1.0) |
| TFB | 9 | 72.5±7.0 | 67.4±2.1 | 6.75±0.34 | 192±4 |
| | | (2.8) | (11) | (23) | (2.2) |
| MD | 284 | 103±17 | 12.3±3.5 | 0.62±0.08 | 137±14 |
| | | (4.0) | (2.1) | (2.1) | (1.6) |
| PB | 3600 | 149±11 | 19.4±2.4 | 1.80±0.17 | 149±12 |
| | | (5.7) | (3.3) | (6.2) | (1.8) |
| FEN | >11000 | 365±8 | 28.4±4.7 | 5.17±0.55 | 147±5 |
| | | (14) | (4.8) | (18) | (1.7) |
| F/PB | >11000 | 409±13 | 26.8±3.6 | 2.58±0.17 | 169±5 |
| | | (16) | (4.5) | (8.9) | (2.0) |

SOURCE: Adapted from refs. 22, 24 and 35.

[a] FS: susceptible strain, MPA: methyl parathion-selected strain, TFB: teflubenzuron-selected strain, MD: insecticide pressure-relaxed field strain, PB: piperonyl butoxide (PB)-selected strain, FEN: fenvalerate-selected strain, and F/PB: fenvalerate plus PB-selected strain.

[b] RR: resistance ratio for fenvalerate.

[c] Mean±SE of two to four replicates.

[d] MROD: methoxyresorufin *O*-demethylase (pmol/min/mg protein), EROD: ethoxyresorufin *O*-deethylase (pmol/min/mg protein), ECOD: ethoxycoumarin *O*-deethylase (nmol/min/mg protein), and AE: aldrin epoxidase (pmol/min/mg protein).

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acetylcholinesterase sensitivity to these compounds (*23,24*). Available data suggest that detoxication by microsomal monooxygenases was not a significant mechanism for resistance to organophosphorus insecticides in DBM. Moreover, DBM resistance to some organophosphorus insecticides is rather unstable (*20*), in contrast to pyrethroid resistance. In addition, a fenvalerate resistant strain of DBM (resistance ratio 770) had only 7-fold cross resistance to methyl parathion, further supporting the conclusion that no major common mechanism of resistance exists between organophosphorus insecticides and pyrethroids. This explains why pyrethroids were enthusiastically accepted when they first appeared in the market in mid-1970s, and why farmers often go back to organophosphorus compounds when pyrethroid resistance occurs. We have also examined the effect of enhanced microsomal oxidation on bioactivation of organophosphorus insecticides and the results will be discussed in a later section.

**Components of Microsomal P450-dependent Monooxygenases.** Binding spectra of microsomal cytochrome P450. Due to their small size, whole larvae of DBM are used to prepare the microsomes. Cytochrome P450 thus prepared is especially unstable. Nevertheless, we have obtained through use of freshly prepared microsomes consistent and stable CO-P450 binding spectra with minimal P420 absorption. The first C0-450 difference spectrum recorded for diamondback moth larvae (Fig. 1) resembles that of other insects and mammals (Fig. 2), with a slight P420 peak and $\lambda$ max of 450 nm. The specific content of microsomal P450 from diamondback moth larvae (0.10-0.19 nmol/mg protein; whole larval homogenate) (Table II) is somewhat lower than that reported for house fly abdomen (*25*) and midguts of some lepidopterans (*26*).

Earlier reports showed that type I spectra are only associated with resistant house fly and several other insects which had high microsomal monooxygenase activities (*27,28*). Subsequent studies, however, revealed type I binding in susceptible house fly as well (*29*). In this work microsomes from both susceptible and resistant DBM were able to produce barely detectable type I spectra with (-)-menthol, giving a peak at 385-390 nm and a trough at approximately 420 nm (Fig. 3).

Binding of microsomes from DBM larvae with pyridine resulted in a type II difference spectrum, with a peak at 420-430 nm and a wide trough between 370-410 nm (Fig. 3). In house fly, type II spectra formed with *n*-octylamine were reported to occur in two forms, one with a double trough (420 and 394 nm) in a susceptible strain of house fly, and the other with a single trough (390 nm) in both resistant and high monooxygenase house fly strains (*30*). The type II spectrum with *n*-octylamine displayed by DBM larvae

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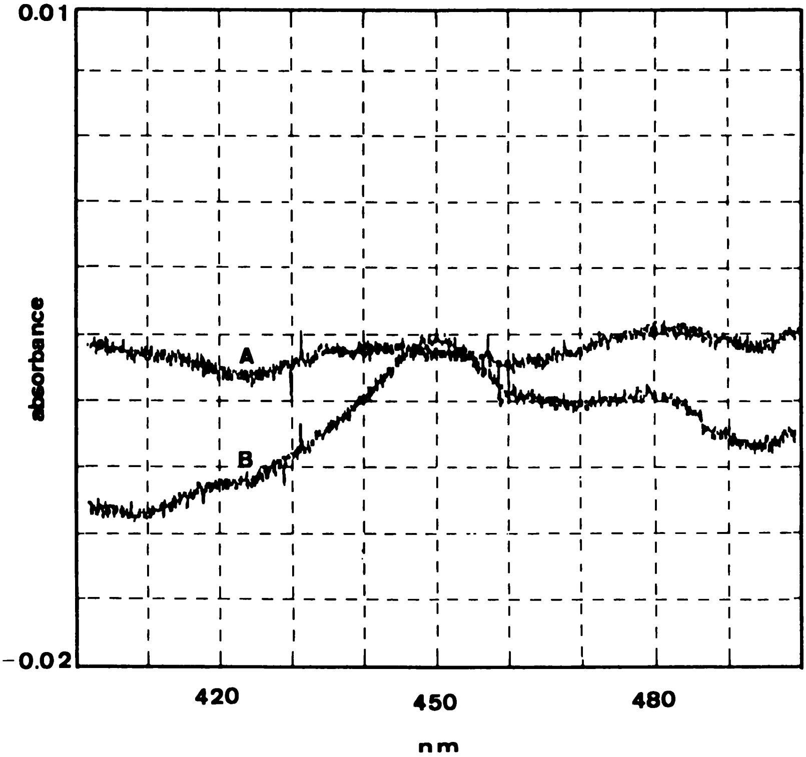

Fig. 1. CO-reduced cytochrome P450 spectrum of microsomes from fourth instar larvae of susceptible diamondback moth. A: baseline, B:difference spectrum.

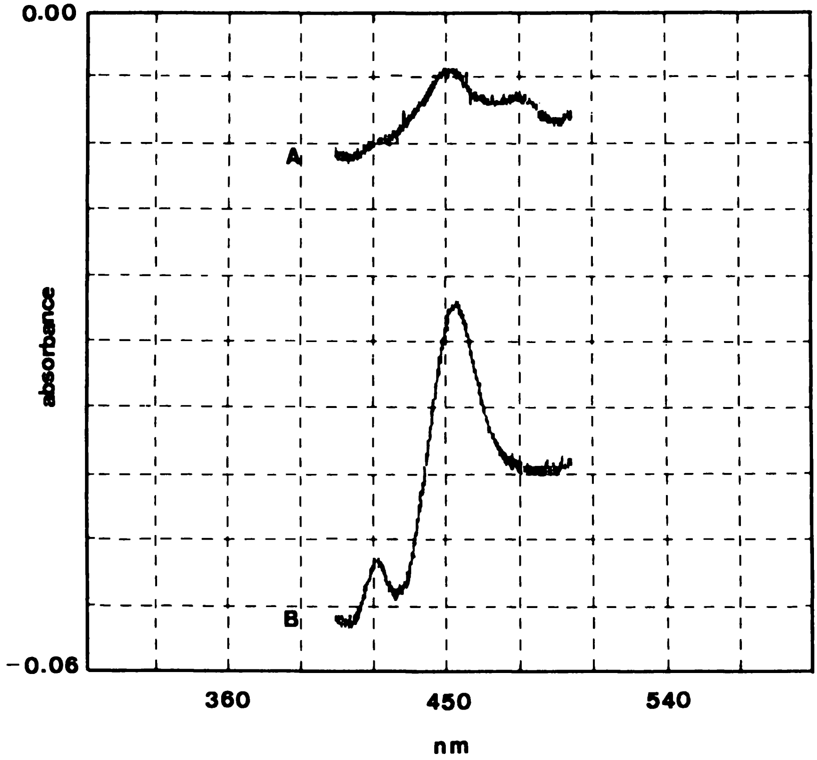

Fig. 2. CO-reduced cytochrome P450 spectra of microsomes from diamondback moth (A) and mouse liver (B).

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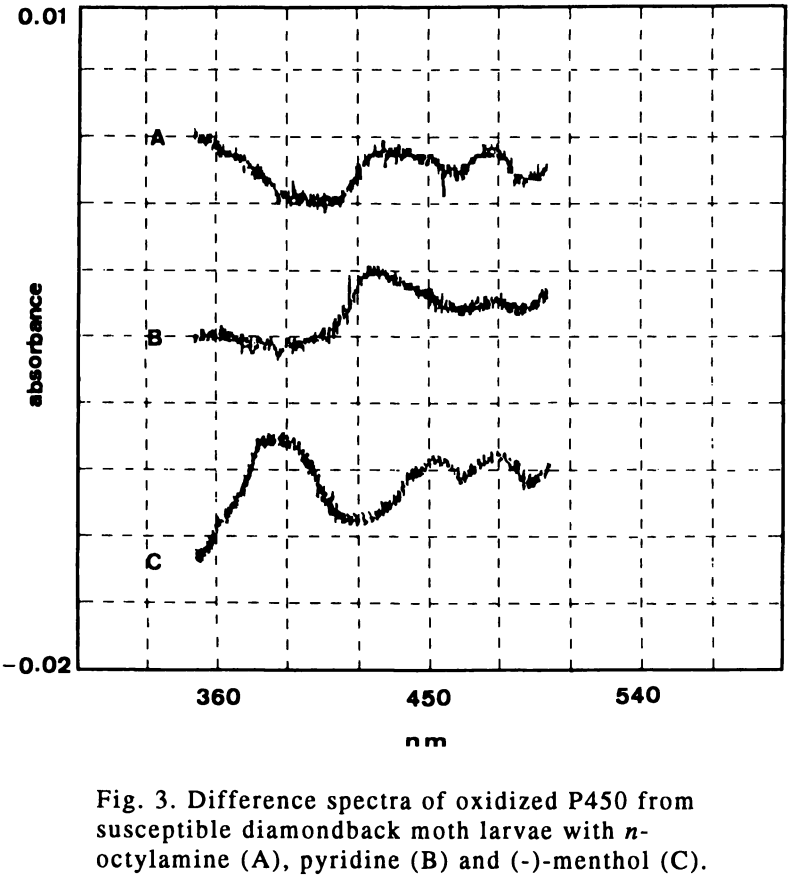

Fig. 3. Difference spectra of oxidized P450 from susceptible diamondback moth larvae with *n*-octylamine (A), pyridine (B) and (-)-menthol (C).

(susceptible and resistant) was too small in magnitude to resolve these features (Fig. 3).

Table II. Components of microsomal P450-dependent monooxygenases of several strains of diamondback moth larvae

| Strain[a] | Cytochrome P450 | Cytochrome b5 | Cytochrome c reductase |
|---|---|---|---|
| | pmol/mg protein | | nmol/min/mg protein |
| FS | 99.5±4.2[b]<br>(1.0) | 43.8±1.6<br>(1.0) | 12.9±1.2<br>(1.0) |
| FEN | 144±13<br>(1.5) | 68.1±2.7<br>(1.6) | 46.7±3.6<br>(3.6) |
| TFB | 190±16<br>(1.9) | 57.1±1.9<br>(1.3) | 25.9±0.5<br>(2.0) |
| ABM | 126±10<br>(1.3) | 42.0±1.6<br>(1.0) | 14.6±2.0<br>(1.1) |
| LC | 145±16<br>(1.5) | 53.2±1.1<br>(1.2) | 21.7±1.3<br>(1.7) |

[a] FS: susceptible strain, FEN: fenvalerate-selected strain, TFB: teflubenzuron-selected strain, ABM: abamectin-selected strain, and LC: resistant field strain.

[b] Mean±SE of three to five experiments.

Qualitative and quantitative differences of components in microsomal P450-dependent monooxygenases. The CO-reduced cytochrome P450 binding spectrum of fenvalerate resistant DBM had a λ max of 448-449nm, as compared with a λ max of 450 nm for susceptible DBM (Fig. 4). This shift in absorption maximum was consistently observed and thus considered a unique feature of P450s in the fenvalerate resistant DBM. A similar shift was previously reported in diazinon/dimethoate resistant house fly (*31*). However, in a recent study using two organophosphorus resistant and four pyrethroid resistant strains of house fly, only one pyrethroid resistant strain exhibited an apparently low λ max (450 nm) as compared with the susceptible strain (452.5 nm) (*32*). While differences in microsomal preparation procedures and instrumentation used may obliterate slight spectral changes, an obvious and consistent shift of the absorption maximum for CO-

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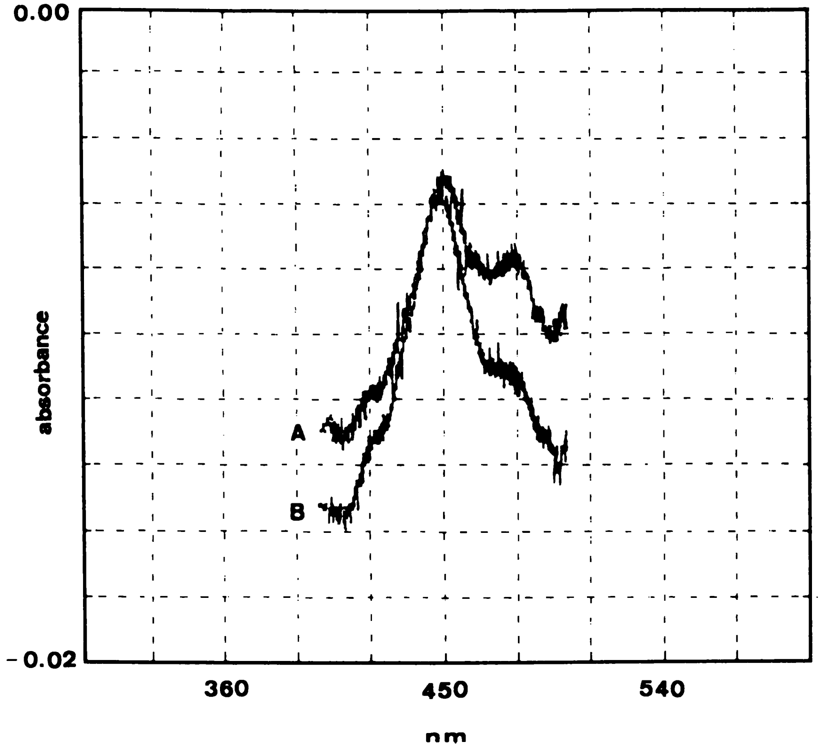

Fig. 4. CO-reduced cytochrome P450 spectra of microsomes from a susceptible (A) and a fenvalerate resistant (B) strain of diamondback moth larvae.

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P450 spectrum very possibly represents a significant change in relative abundance of P450 isozymes. Thus P450s of fenvalerate resistant DBM are probably qualitatively different from P450s of susceptible DBM.

Elevated levels of the three major components of microsomal monooxygenases have been detected in resistant house fly (*32,33*), and all three have been suggested to play a role in insecticide resistance (*32*). In our work, specific activities of cytochrome P450, cytochrome b5 and cytochrome c reductase are 1.5-,1.6- and 3.6-fold higher in fenvalerate resistant DBM than in the susceptible DBM (Table II).

## Benzoylphenyl Urea Resistance

Surveys carried out in 1986 and 1987 before the introduction of benzoylphenyl ureas into Taiwan showed that DBM larvae with high levels of resistance to organophosphorus, carbamate and pyrethroid insecticides did not have cross resistance to either teflubenzuron or chlorfluazuron (*34*).

**Occurrence.** Diflubenzuron, the first commercialized benzoylphenyl urea, is not effective toward even the susceptible DBM, and therefore it has never been recommended for DBM control in Taiwan; yet, field DBM were moderately resistant to this chitin synthethsis inhibitor before other benzoylphenyl ureas were used (*34*). Six months after a full scale application of teflubenzuron in Taiwan, field DBM larvae exhibited a general increase of tolerance to this insecticide, and larvae collected from one location where apparent control failures had been observed, had 31-fold resistance (*35*). Since then, control failure with teflubenzuron and subsequently with chlorfluazuron have been observed throughout the island. Moderate benzoylphenyl urea resistance has also been reported in DBM from Thailand (*36*), Malaysia (*15, 37*) and Japan (*36*). This resistance appears quite stable (*36*), and laboratory selection of a moderately resistant field population resulted in rapid development of high levels of resistance (*37*).

**Resistance Mechanisms.** Due to the recent development of benzoylphenyl urea resistance in DBM, there is only scanty and sometimes controversial information on resistance mechanisms.

**Non-metabolic Mechanisms.** Compared with teflubenzuron, diflubenzuron is 1000-fold less toxic against the susceptible DBM; and PB-synergized diflubenzuron is still ca. 300-fold less toxic (*38*) . This implies the posssible existence of non-metabolic resistance mechanisms. A recent study demonstrated that a large part (67%) of applied diflubenzuron and teflubenzuron was rapidly

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excreted from the highly tolerant beet army worm, *Spodoptera exigua* (*39*). We are unable to speculate now if target (chitin synthesis processes) insensitivity would be found involved in DBM resistance to this group of compounds.

**Metabolic Mechanisms.** Addition of the synergist PB completely restored the teflubenzuron susceptibility of the above mentioned field resistant strain of DBM (*35*). By contrast, a hydrolytic mechanism was not detected in synergist studies (*34*). Thus, increased detoxication mediated by microsomal P450-dependent monooxygenases has been proposed as the major mechanism for benzoylphenyl urea resistance in this insect (*34*, *35*). This accounts for the observation that there is no significant cross resistance between organophosphorus insecticides and the chitin synthesis inhibitors (*34*). Activities of this detoxifying enzyme toward some model substrates are 7 to 28-fold higher in teflubenzuron resistant strains (resistance ratios 18 to 36-fold) than those in a susceptible strain (Table III) (*35*). Differences in epoxidation between susceptible and resistant strains are much smaller than in *O*-dealkylation and aryl hydroxylation. Again, attempts to detect *in vitro* oxidation of teflubenzuron in DBM by measuring substrate disappearance with high performance liquid chromatography and substrate-dependent NADPH consumption have been unsuccessful.

**Cytochrome P450 Isozymes.** Enhanced microsomal detoxication has been proposed as the major resistance mechanism in DBM for both pyrethroids and benzoylphenyl ureas. And yet, DBM with >3600-fold resistance to fenvalerate and 5 to 16-fold increase of microsomal *O*-dealkylase activities (*22*) did not have any significant cross resistance to teflubenzuron (*35*). Also, selection of DBM with teflubenzuron did not result in significant cross resistance to permethrin and fenvalerate either (*38*). We thus propose that different forms of cytochrome P450s in DBM larvae are involved in the detoxication of these two groups of insecticides. This is supported by the observation that while the CO-reduced P450 spectrum for microsomes from a fenvalerate resistant strain had a 1-2 nm shift of $\lambda$ max (Fig. 2), the spectrum of a teflubenzuron resistant strain retained a $\lambda$ max of 450 nm, just like the susceptible strain. Contents of P450, cytochrome b5 and cytochrome c reductase of the teflubenzuron-selected TFB strain and the LC strain (collected from the field with similar resistance level) were higher than those of the susceptible strain, and their pattern of changes is somewhat different from that of the fenvalerate resistant strain (Table II).

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Table III. Microsomal monooxygenase activities of diamondback moth larvae with differnt levels of teflubenzuron resistance

| | | Specific activity[c,d] | | | | |
|---|---|---|---|---|---|---|
| Strain[a] | RR[b] | MROD | EROD | ECOD | AE | AHH |
| FS | 1 | 26.0±1.3<br>(1.0) | 5.9±1.8<br>(1.0) | 0.29±0.03<br>(1.0) | 85.6±4.2<br>(1.0) | 28.1±1.5<br>(1.0) |
| MD | 2 | 103±17<br>(4.0) | 12.3±3.5<br>(2.1) | 0.62±0.08<br>(2.1) | 145±5<br>(1.7) | 46.7±5.8<br>(1.7) |
| FEN | 3 | 365±8<br>(14) | 28.4±4.7<br>(4.8) | 5.17±0.55<br>(18) | 147±5<br>(1.7) | _ |
| HH | 5 | 160±11<br>(6.2) | 13.5±0.9<br>(2.3) | 1.05±0.05<br>(3.6) | 181±1<br>(2.1) | _ |
| FSR | 18 | 508±18<br>(20) | 57.5±3.7<br>(9.8) | 6.35±0.18<br>(22) | 264±7<br>(3.1) | 220±11<br>(7.8) |
| LC | 31 | 400±9<br>(15) | 39.8±2.7<br>(6.8) | 5.20±0.40<br>(18) | 253±4<br>(3.0) | _ |
| MDR | 36 | 725±7<br>(28) | 67.4±2.1<br>(11) | 6.75±0.34<br>(23) | 192±4<br>(2.2) | 151±7<br>(5.4) |

SOURCE: Adapted from refs. 34 and 35.

[a] FS: susceptible strain, MD: insecticide pressure-relaxed strain, FEN: fenvalerate-selected strain, HH and LC: two field strains, FSR and MDR: two teflubenzuron-selected strains.

[b] RR: resistance ratio for teflubenzuron.

[c] Mean±SE of two to four replicates.

[d] MROD: methoxyresorufin *O*-demethylase (pmol/min/mg protein), EROD: methoxyresorufin *O*-deethylase (pmol/min/mg protein), ECOD: ethoxycoumarin *O*-deethylase (nmol/min/mg protein), AE: aldrin epoxidase (pmol/min/mg protein), and AHH: aryl hydrocarbon hydroxylase (pmol/min/mg protein).

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## Bioactivation of Organophosphorus Insecticides and Association with Pyrethroid/Benzoylphenyl Urea Resistance

One question that arises with regard to the above discussion is that enhanced activity of microsomal monooxygenases in pyrethroid resistant and benzoylphenyl urea resistant DBM may make some organophosphorus compounds more toxic through bioactivation. Using gas chromatrography, we observed neither disappearance of methyl parathion nor production of methyl paraoxon. Subsequently, we studied this by measuring in DBM homogenates the extent of methyl paraoxon formation from addedmethyl parathion through the inhibition of electric eel acetylcholinesterase (AChE) activity (*40*). In absence of added NADPH, a 13% inhibition of AChE was detected as the control. The susceptible and methyl parathion-selected strains were similar in their capacity to oxidize this organophosphorus insecticide (Table IV). Yet, ca. 2 to 3-fold higher rate of bioactivation of methyl parathion was observed in both pyrethroid and benzoylphenyl urea resistant strains. When both NADPH and reduced glutathione were provided, DBM larval homogenate activated (through oxidation) and detoxified

Table IV. Oxidative activition of methyl parathion by larval homogenates of several strains of diamondback moth

| Strain[a] | % Inhibition of acetylcholinesterase[b,c] |
|---|---|
| FS | 32±5 |
| MPA | 39±4 |
| FEN | 56±1 |
| TFB | 65±1 |

[a] FS: susceptible strain, MPA: methyl parathion-selected strain, FEN: fenvalerate-selected train, andTFB: teflubenzuron-selected strain.

[b] Four units of eel acetylcholinesterase were used. The concentration of methyl parathion was $10^{-4}$ M.

[c] Mean±SE of three replicates.

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(through conjugation) methyl parathion in an independent manner (data not shown), and conjugation was ca. 1000-fold faster than oxidation (*23*). Thus, despite the active detoxication via glutathione transferase, organophosphorus insecticides may be activated at higher rates so as to cause more AChE inhibition in pyrethroid and benzoylphenyl urea resistant DBM. This helps explain, in part, farmers' practice of alternating pyrethroids/ benzoylphenyl ureas with organophospharus compounds for DBM control.

## Glutathione Transferase and Pyrethroid/Benzoylphenyl Urea Resistance

Recently, glutathione transferase (GST) was implicated in pyrethroid resistant cotton bollworm, *Spodoptera littoralis*, through a marked synergism of endosulfan and pyrethroid toxicity by diethylmaleate (*41*). Based on the effect of this same synergist on amounts of polar metabolites produced, conjugation was reported to play an important role in the detoxication of teflubenzuron in *Spodoptera exigua* larvae (*39*). We thus compared the activities of GST toward 1, 2-dichloronitrobenzene (DCNB), methyl parathion, fenvalerate and teflubenzuron in several strains. In these strains there was little cross resistance between methyl parathion and fenvalerate or between these two compounds and teflubenzuron (Table V).

Table V. Resistance to some insecticides in several strains of diamondback moth larvae

| | Resistance ratio | | |
|---|---|---|---|
| Strain[a] | Methyl parathion | Fenvalerate | Teflubenzuron |
| FS | 1 | 1 | 1 |
| MPA | 455 | 4.7 | 1.5 |
| FEN | 7 | 770 | 3.7 |
| TFB | 30 | 8.8 | >100 |

[a] FS: susceptible strain, MPA: methyl parathion-selected strain, FEN: fenvalerate-selectedstrain, and TFB: teflubenzuron-selected strain.

[b] LC50s of FS strain are: methyl parathion, 0.06 mg/ml ; fenvalerate, 0.027 mg/ml; and teflubenzuron, 0.071 ug/ml.

However, moderate cross resistance from teflubenzuron to methyl parathion was observed. While all resistant strains had considerably higher GST activity toward both DCNB and methyl parathion than susceptible strain, TFB strain had the highest level of GST (Table VI). Why such a high level of GST accompanies a high level of microsomal P450 monooxygenases in teflubenzuron resistant DBM and why this high GST activity measured *in vitro* is not associated with high methyl parathion resistance in the same strain are unclear.

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Table VI. Glutathione transferase activities of several strains of diamondback moth larvae with different levels of methyl parathion resistance

| | | Specific activity[c,d] nmol/min/mg protein | |
|---|---|---|---|
| Strain[a] | RR[b] | DCNB | Methyl parathion |
| FS | 1 | 84.4±5.5<br>(1.0) | 28.6±0.9<br>(1.0) |
| MPA | 455 | 156±9<br>(1.8) | 112±2<br>(3.9) |
| FEN | 7 | 119±7<br>(1.4) | 62.5±9.9<br>(2.2) |
| TFB | 30 | 183±15<br>(2.2) | 119±5<br>(4.2) |

[a]FS: susceptible strain, MPA: methyl parathion-selected strain, FEN: fenvalerate-selected strain, and TFB: teflubenzuron-selected strain.

[b]RR: resistance ratio for parathion.

[c]Mean±SE of two to three replicates.

[d]DCNB: 1,2-dichloronitrobenzene.

## Concluding Remarks

The rapid selection of elevated microsomal P450 monooxygenases that detoxify pyrethroids and new forms of cytochrome P450s to cope with benzoylphenyl ureas demonstrated the versatile nature of this detoxifying enzyme system in DBM. Development of new chemicals for DBM control should take this into consideration.For example, partial synergism of abamectin by PB has been observed in laboratory-selected strain showing >100-fold resistance(data not shown); there is a small increase of components for monooxygenases (Table II) without a concomitant P450 spectrum change. Future studies in DBM need to address the coupling of primary oxidation and secondary conjugation., i. e., the relative distribution and localization of enzymes for these two steps (*42*) in order to elucidate the complicated phenomena of insecticide resistance in this pest.

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## Chapter 13

# Insecticide Resistance in the Tufted Apple Bud Moth, a Polyphagous Lepidopteran

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Azinphosmethyl resistance in the tufted apple bud moth (TABM), a tortricid pest of apple, provides a significant evolutionary and pest management problem. Current biochemical evidence using synthetic substrates indicates that higher glutathione transferase activity is associated with increased resistance in TABM field populations. Although resistance varies greatly among different habitats occupied by TABM, an allozyme study suggests that moths in these habitats experience regular interhabitat gene flow. We have begun to identify glutathione transferase genes in TABM using a heterologous *Drosophila* probe.

Patterns of insecticide resistance in pest populations are complex. Factors influencing resistance range from pest life history (effective population size, voltinism, feeding habits, diet breadth, dispersal capabilities) to crop variables (annual vs. perennial, field size, adjacent vegetation type, mix of cultivars) to the pesticide used (dosage, persistence, application method, target site, detoxication route) to insect genetics (gene structure, population genetics, pleiotropy) (*1*, *2*). Clear understanding of the evolution of resistance and, more importantly, the ability to predict the likelihood of resistance require that as many of these parameters as possible be addressed.

We have begun to unite population biology with traditional toxicological techniques and to expand this into an investigation of the molecular evolution of detoxication in a leaf-rolling apple pest, the tufted apple bud moth, *Platynota idaeusalis* (Lepidoptera: Tortricidae). Resistance to organophosphate insecticides, particularly azinphosmethyl, has become an economic problem for Pennsylvania apple producers over the past 15 years. Resistance influences the choice of pesticides available, and this choice has an impact on the integrated pest management (IPM) system currently in place in Pennsylvania apple orchards. Sufficient field work has been conducted to make this plant-insect combination attractive for further study.

## Plant–Insect System

Tufted apple bud moth (TABM) is a bivoltine species with a host record of at least 17 plant families (*3*, *4*, *5*). TABM feeds on most orchard crops, but, in Pennsylvania

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and along the eastern seaboard, has an economically significant effect only on apple. Leafrollers are not traditionally classified as primary pests, but TABM frequently ties leaves directly to developing fruit. The result is feeding damage on the fruit, which has a noticeable economic impact (*6*).

The standard control method for many years has been various organophosphate insecticides, especially azinphosmethyl, applied as a series of cover sprays. A successful IPM program targeted at the European red mite, *Panonychus ulmi*, has been in place for nearly 20 years in Pennsylvania orchards. This program relies on the tolerance of the major mite predator, *Stethorus punctum* (Coleoptera: Coccinellidae), to these organophosphate insecticides. IPM has led to a substantial reduction in pesticide application.

Azinphosmethyl resistance was first documented in Pennsylvania TABM in 1984 (*7*). Comprehensive surveys of managed and unmanaged habitats demonstrated a mosaic pattern of resistance in different crops (*8*), and these interhost differences appear stable over a span of at least four years. On a larger scale, substantial heterogeneity is evident among TABM populations from apple orchards all along the east coast of the United States (*9*). The economic importance of this pest has led to intensive study of TABM biology, including measurement of adult dispersal (*5*, *10*) and larval growth and development on different hosts (*11*).

## TABM Resistance to Azinphosmethyl

Initially, bioassays, both via direct spray and leaf residue, were used to establish levels of resistance to most of the common insecticides in Pennsylvania orchards (*12*). These results indicate a roughly 20-fold level of resistance to azinphosmethyl in neonate larvae. Minimal cross resistance is evident in field populations to either methomyl or fenvalerate (resistance ratios of contact $LC_{50}$ values were 2.4 and 4.3, respectively; *12*), the two most commonly applied non-organophosphate materials in Pennsylvania orchards. Methods of assaying resistance levels from adult males collected on sex pheromone traps demonstrated somewhat lower resistance ratios, but the results were generally consistent with bioassays of neonates (*8*, *9*).

We are investigating potential biochemical mechanisms of resistance to azinphosmethyl with the goal of identifying the underlying genetic basis of this trait. Our initial approach has been to measure detoxication capabilities in adult male TABM using standard synthetic substrates for esterases, glutathione transferases, and cytochrome P450 monooxygenases. We have also determined relative acetylcholinesterase activities from the same populations.

**Sample Collections.** All assays were done on adult male moths captured in sex pheromone traps in Adams County, Pennsylvania. Adult males were chosen because of their availability; adult females and immature stages are difficult to find in unmanaged habitats, so adult males were our only option to obtain necessary sample sizes. Pheromone traps were placed in two commercial apple orchards separated by approximately 10 km, with an intervening mountain range. A corresponding woods site, representing unmanaged habitat, was selected within 1-2 km of each apple site, and a third, more isolated (over 20 km from either apple site and over 5 km from any commercial orchards) woods site was also identified and trapped. Moths were trapped during the peak flight period for each of the two generations in 1990. Moths were removed from adhesive traps while still alive and frozen in liquid nitrogen. Samples were later transferred to a -80° C freezer and held until needed for analysis. A subset of moths from each habitat was bioassayed using a dorsally applied topical dose of 282 μg/g body weight, the $LD_{99}$ of a susceptible laboratory population. Mortality levels in generation 1, 1990, ranked the populations from most resistant to most susceptible as follows: Apple 1 (42% mortality), Apple 2 (52%), Woods 1

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(66%), Woods 2 (87%), and Isolated Woods (97%). Both Apple sites experienced 75% mortality in generation 2, while Woods 2 showed 93% mortality. Sample sizes were insufficient to obtain a reliable response to the diagnostic dose for Woods 1 and Isolated Woods in generation 2.

**Target Site Assays.** Acetylcholinesterase (AChE) $K_m$ and $V_{max}$ levels for acetylthiocholine were determined by the Ellman method (*13*) as measures of target site activity , and no differences were observed among populations (Apple 1: $K_m$ = 21 µM, $V_{max}$ = 77 µmole $min^{-1}$ mg $protein^{-1}$ ; Woods 1: $K_m$ = 28 µM, $V_{max}$ = 83 µmole $min^{-1}$ mg $protein^{-1}$ ; Apple 2: $K_m$ = 25 µM, $V_{max}$ = 125 µmole $min^{-1}$ mg $protein^{-1}$ ; Woods 2: $K_m$ = 22 µM, $V_{max}$ = 125 µmole $min^{-1}$ mg $protein^{-1}$ ). Eserine inhibition (5 µM final concentration) also failed to discriminate between the AChE of susceptible and resistant TABM populations (Table I).

**Table I. Summary of Target Site Inhibition and Detoxication Capabilities from Five Field Populations of Tufted Apple Bud Moth[1]**

| *Population* | *AChE Inhibition*[2] | *EST*[3] | *GST*[4] | *P450*[5] |
|---|---|---|---|---|
| Apple 1 | 40.6 a | 256 ± 19 ab | 47.6 ± 5 a | 63.5 ± 2 ab |
| Woods 1 | 46.2 a | 321 ± 30 a | 29.7 ± 3 bc | 73.3 ± 3 a |
| Isolated Woods | | 100 ± 20 c | 19.8 ± 2 c | 45.3 ± 7 bc |
| Apple 2 | 40.8 a | 310 ± 34 a | 39.4 ± 4 ab | 31.1 ± 5 c |
| Woods 2 | 35.9 a | 209 ± 19 b | 23.1 ± 2 c | 34.5 ± 7 c |

[1] Means (± S.E.) within a column followed by same letter are not significantly different at $P = 0.05$ (Tukey mean separation test)

[2] Per cent of uninhibited control activity remaining

[3] Hydrolysis of α-naphthyl acetate; µmole $min^{-1}$ mg $protein^{-1}$

[4] Conjugation of 3,4-dichloronitrobenzene; µmole $min^{-1}$ mg $protein^{-1}$

[5] *O*-demethylation of *p*-nitroanisole; nmole $min^{-1}$ mg $protein^{-1}$

**Detoxication Enzyme Assays.** Significant differences between resistant and susceptible moths from Apple 2 and Woods 2 in esterase hydrolysis of α-naphthyl acetate were consistent with differences observed between a laboratory susceptible and an azinphosmethyl-selected resistant colony (*14*). Further comparisons among habitats and across generations demonstrate that there is variation in esterase activity. However, this variation does not always coincide with levels of azinphosmethyl resistance. Apple 1 and Woods 1 do not differ significantly in esterase activity.

Cytochrome P450 monooxygenase (P450) activity was measured by *O*-demethylation of *p*-nitroanisole (*15*) and aniline hydroxylation (*16*). No hydroxylation activity was observed in adult TABM. There are no significant differences between resistant and susceptible populations in *O*-demethylation activity at either site, and neither resistant population is significantly different from the Isolated Woods population (Table I).

In contrast to the inconsistent relationships between esterase or P450 activities and insecticide resistance, we found a strong direct correlation between glutathione transferase (GST) activity and population resistance levels. GST activity was determined spectrophotometrically by conjugation of 3, 4-dichloronitrobenzene (*17*). Resistant populations consistently exhibited significantly higher GST activity levels than did the corresponding susceptible populations (Table I).

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A role for GST in azinphosmethyl metabolism has been proposed for a variety of arthropod species (*18-21*), including TABM (*22*). Since the primary target for field application of azinphosmethyl against TABM is young larvae, we plan to examine GST levels in neonates, although population comparisons will again be limited to strains from apple orchards and a susceptible laboratory colony. It is important to note that substantial heterogeneity in detoxication abilities exists among TABM populations in a relatively restricted geographic range. We are clearly not at a point where we may confidently identify a single enzyme system as the basis for azinphosmethyl resistance. However, we feel that GST plays an important role and will prove an interesting subject for further study.

## Molecular Genetics of Glutathione Transferase

Recent studies (*23,24*, see chapter by Cochrane et al.) have identified and characterized GST genes from two insects, *Drosophila melanogaster* and *Musca domestica*. No GST genes have yet been extracted from a phytophagous insect. Characterization of such genes would be of obvious interest both from pesticide resistance and host plant utilization perspectives. We are attempting to use a *Drosophila melanogaster* GST1-1 (*23*) probe to identify homologous genes from TABM. If sufficient sequence conservation exists, this probe will be used to screen a TABM genomic library that we have constructed in EMBL-4.

The major detoxication enzyme systems are all members of multigene families. This type of gene organization may facilitate the diversification of function in an evolutionary sense, in that one or more of the gene copies may accumulate mutations that would be subject to negative selection pressure if they were single-copy genes. Much work remains to properly elucidate detoxication gene organization in insects, but this is a critical line of investigation if we are to ever resolve some of the basic issues in insect-xenobiotic interactions (*25,26*).

## TABM Population Structure and Resistance

Insecticide resistance clearly is selected for and spreads in a population of insects. Thus, a complete explanation for the evolution of resistance requires understanding the population genetic structure of the pest species (*27-29*). This is of particular interest in what, to our eye, is a widely polyphagous insect, that is, TABM. It is imperative to rule out the possibility that this is actually a complex of unidentified sibling species, each specializing on a subset of the total host range and not exchanging genes (e.g. *30*). Even if this is a single, polyphagous species, the potential exists for TABM to be structured into subpopulations on the basis of host plant use or geographic features. Either of these scenarios would have an effect on the opportunity for gene flow among populations and, therefore, influence the spread of resistance alleles.

TABM populations in Adams County, Pennsylvania, display heterogeneity in the azinphosmethyl resistance phenotype. Knight and Hull (*5*) used a mark-recapture study of adult male TABM to quantify dispersal in a multi-host habitat. Female movement was quantified by releasing adult females marked with rubidium chloride and recovering egg masses from orchard leaves (*9*). These ecological approaches showed that adult TABM have the ability to disperse over distances separating the paired apple-woods sites in this study. A further, unquantified component of dispersal is the ballooning behavior of larvae.

An allozyme study of TABM population structure revealed no evidence of population structure either on a host-related basis or on a geographical scale (McPheron, B.; McLinden, R.; Barrett, B.; Hull, L., unpublished data). Adult moths were captured on pheromone traps from five habitats in both generations during 1989. Allele frequencies for ten polymorphic enzymes were accumulated and analyzed. Only 4 of 77 comparisons of allele frequencies across sites or generations were significant, and no pattern of allelic heterogeneity was evident, consistent with the interpretation that this is a single, polyphagous species that experiences relatively high levels of gene flow among habitats. However, this population structure is not consistent with the temporally stable patterns of azinphosmethyl resistance among these same habitats. We are investigating TABM population structure with other, potentially more sensitive, genetic markers. Clearly, the best tool for resolving the apparent genetic panmixis and the definite phenotypic (resistance) differences among populations is the actual gene responsible for azinphosmethyl detoxication (cf. the approach of Raymond et al. (*28*)).

## Prospects for a Synthetic Approach to Resistance

A comprehensive attack on the problem of insecticide resistance will unite economic entomology, insect ecology, toxicology, population genetics, and molecular biology. Azinphosmethyl resistance remains stable in TABM populations using managed tree fruit crops in southcentral Pennsylvania, despite reduced reliance on this compound (due primarily to resistance problems; Hull, L., Rajotte, E., personal communication). We plan to verify the role of GST in azinphosmethyl detoxification and isolate and characterize the genetic mechanism involved. Identification of a resistance allele will permit an array of studies that have been difficult or impossible in the past (*29,31*), including elucidation of the spread of resistance among habitats, the role of alternative chemicals, natural or synthetic, as a selective force on resistance alleles, the possibility of an induction mechanism in detoxication gene expression, and the potential fitness costs of detoxication ability. This multidisciplinary approach will give us maximum information on the evolution of this system while also preparing us to more effectively manage this crop pest.

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# Chapter 14

# Plant-Allelochemical-Adapted Glutathione Transferases in Lepidoptera

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Glutathione transferases metabolized toxic allelochemicals, including α,β-unsaturated carbonyl compounds, isothiocyanates and organothiocyanates in lepidopterous insects. These transferase activities in the specialist velvetbean caterpillar are lower than in the generalist fall armyworm; the activity toward the isothiocyanates in the crucifer-adapted cabbage looper was 2- to 6-fold higher than that in the fall armyworm. Host plants such as crucifers and umbellifers, and allelochemicals such as coumarins, indoles, flavonoids, isothiocyanates and monoterpenes induced glutathione transferases in these insects. The highly polyphagous Lepidoptera, fall armyworm and corn earworm, possessed multiple glutathione transferases containing six and four isozymes, respectively, whereas the more specialized Lepidoptera, tobacco budworm, cabbage looper and velvetbean caterpillar, had a single form of the enzyme. The results suggest that glutathione transferases play an important role in allelochemical resistance in phytophagous Lepidoptera.

Glutathione transferases are a group of detoxication enzymes catalyzing the conjugation of glutathione (GSH) with various xenobiotics possessing a reactive electrophilic center (1). Since the conjugates are subsequently transformed in animals to give excretable mercapturic acids (2), glutathione-dependent conjugation has been regarded as an important detoxication mechanism in insects as well as in mammals. These enzymes perform a variety of reactions including (a) the S-alkylation of

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GSH by alkyl halides and related compounds; (b) the replacement of labile aryl halogen or nitro groups by GSH; (c) the replacement of labile aralkyl halogen by GSH; (d) the addition of GSH to various epoxides; (e) the addition of GSH to α,β-unsaturated compounds including aldehydes, ketones, lactones, nitriles and nitro compounds; and (f) the O-alkyl and O-aryl conjugation of phosphorothioates and phosphates with GSH (3). Because of their broad substrate specificities, glutathione transferases are responsible for the detoxication of numerous toxicants.

Glutathione transferases are important in the phase II metabolism of reactive metabolites formed by microsomal oxidations. They are also important in the phase I metabolism of organophosphorus insecticides (OP) and are believed to play an important role in OP resistance in insects (4,5). Our recent work has also shown that glutathione transferases are involved in the metabolism of potentially toxic allelochemicals including α,β-unsaturated carbonyl compounds, isothiocyanates and organothiocyanates in lepidopterous insects (6-8). Evidence accumulated so far indicates that glutathione transferases play important roles in the detoxication of allelochemicals and may be related to host plant range in phytophagous insects.

This paper reviews the current knowledge of the biochemical interactions between glutathione transferases and allelochemicals in lepidopterous species and presents experimental evidence suggesting that glutathione transferases play a significant role in allelochemical resistance in these insects.

## Detoxication of Toxic Allelochemicals by Glutathione Transferases in Lepidoptera

Many allelochemicals possess structural requirements to serve as substrates for glutathione transferases. For example, the α,β-unsaturated carbonyl moiety is commonly found in plant constituents such as coumarins, quinones, terpenoids, cardenolides, alkaloids, etc. We found that the allelochemicals trans-cinnamaldehyde, trans-2-hexenal, trans, trans-2,4-decadienal and benzaldehyde were metabolized by glutathione transferases from the soluble fraction of the midgut homogenates of fall armyworm (FAW, Spodoptera frugiperda) larvae with trans-cinnamaldehyde being a preferred substrate (Table I). Using trans-4-phenyl-3-buten-2-one (TPBO) as a model substrate, it was found that the TPBO transferase activity was different from that toward the model substrate 1,2-dichloro-4-nitrobenzene (DCNB) based on its distribution pattern in the protein fractions isolated by ammonium sulfate fractionation of the cytosol (6).

In addition, plant isothiocyanates were found to be metabolized by glutathione transferase from lepidopterous

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**Table I. Glutathione transferase activity in lepidopterous insects toward plant allelochemicals[a]**

| Substrate | Glutathione transferase (nmol/min/mg protein)[b] | | |
|---|---|---|---|
| | Fall armyworm | Cabbage looper | Velvetbean caterpillar |
| Trans,trans-2,4-decadienal | 4.67 ± 0.70 | | |
| Trans,trans-cinnamaldehyde | 10.05 ± 0.46 | | |
| Benzaldehyde | 3.59 ± 0.67 | | |
| Trans-2-hexenal | 4.08 ± 0.48 | | |
| Allyl isothiocyanate | 95.0 ± 45.0 | 57.0 ± 20.0 | 0 |
| Benzyl isothiocyanate | 80.0 ± 30.0 | 170.0 ± 65.0 | 0 |
| Benzyl thiocyanate | 13.6 ± 1.20 | 16.6 ± 4.00 | 9.20 ± 4.00 |

[a] Adapted from refs. 6-8.
[b] Mean ± SE.

larvae using the midgut soluble fraction as the enzyme source (Table I). Allyl and benzyl isothiocyanates were metabolized by glutathione transferase from the two generalists, the fall armyworm and cabbage looper (CL, Trichoplusia ni), but no activity was detected from the specialist velvetbean caterpillar (VBC, Anticarsia gemmatalis). The generalists, but not the specialist, are adapted to feeding on isothiocyanate-containing crucifers. The transferase activity toward these allelochemicals in the crucifer-adapted cabbage looper was 2- to 6-fold higher than that in the fall armyworm. The transferase system of fall armyworm also metabolized 2-phenylethyl isothiocyanate, but activity can only be observed after induction (7).

Data in Table I also demonstrated that the organothiocyanate allelochemical benzyl thiocyanate was metabolized by glutathione transferase from larvae of three lepidopterous species, the fall armyworm, cabbage looper and velvetbean caterpillar, using the midgut soluble fraction as the enzyme source.

From Table II, it can be seen that these $\alpha,\beta$-unsaturated carbonyl compounds, isothiocyanates and an organothiocyanate were all toxic to the fall armyworm, causing acute toxicity in neonates. Among those tested, benzyl and 2-phenylethyl isothiocyanates were the most acutely toxic to the armyworm. The isothiocyanates and the organothiocyanate were also toxic to other Lepidoptera such as the velvetbean caterpillar and cabbage looper (6-8).

## Purification and Characterization of Glutathione Transferases from Lepidoptera

Glutathione transferases have been purified and characterized in numerous species of insects with particular emphasis on the house fly. Molecular weights of the glutathione transferases studied are within the range 35,000-63,000. They consist of two subunits (homodimers and heterodimers) of molecular weight between 19,000 and 35,000 (9).

In Lepidoptera, Clark et al. (10) were the first to purify glutathione transferase from a lepidopterous insect, the greater wax moth (Galleria mellonella), using affinity chromatography on glutathione-sulfobromophthalein-agarose. Because of its selective ability to bind with glutathione transferase, this enzyme was isolated from this species in substantially pure form. The transferase had a molecular weight of 41,000 with two subunits of $M_r$ 25,000. Its substrate specificity was found to resemble that of the glutathione transferase B from rat liver (11).

Several glutathione transferases from the porina moth (Wiseana cervinata) were purified by affinity chromatography, cation-exchange chromatography and preparative isoelectrofocusing (12). The major

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**Table II. Toxicity of allelochemicals to neonate fall armyworm larvae[a,b]**

| Allelochemical | $LC_{50}$[c] |
|---|---|
| _Trans_,_trans_-2,4-decadienal | 0.033 |
| _Trans_-cinnamaldehyde | 0.077 |
| Benzaldehyde | 0.089 |
| _Trans_-2-hexenal | 0.214 |
| Allyl isothiocyanate | 0.017 |
| Benzyl isothiocyanate | 0.006 |
| 2-Phenylethyl isothiocyanate | 0.006 |
| Benzyl thiocyanate | 0.041 |

[a] Adapted from refs. 6-8.
[b] Groups of 20 neonate larvae were fed artificial diet containing allelochemicals for 24 hr before mortality counts were made.
[c] % in diet (w/w).

transferase was purified to homogeneity by a factor of 530-fold. The molecular weights of these transferases were estimated to be 45,000-50,000. They appeared to be homodimers of either of two types of subunits of $M_r$ 22,800 and 24,600.

Glutathione transferase from the diamondback moth (Plutella xylostella) was purified by either the traditional method involving ammonium sulfate fractionation, gel filtration and hydroxyapatite chromatography or by affinity chromatography (GSH-sulfobromophthalein-agarose). The native glutathione transferase had molecular weights of 45,000-46,500. The purified transferase activity toward DCNB in the insecticide resistant strain was higher than in the susceptible strain (13). That this species possessed a single form of the enzyme was confirmed by Balabaskaran et al. (14) who used ion-exchange chromatography (DEAE-Sephadex A-50 and CM-Sephadex C-50) and adsorption chromatography (Bio-Gel HTP hydroxyapatite). According to these authors, the purified glutathione transferase had an isoelectric point of 9.26 and a molecular weight of 36,400.

Glutathione transferases from larvae of the sugar borer (Diatraea saccharalis) and the Mexican rice borer (Eoreuma loftni) were purified by GSH-affinity chromatography and isoelectric focusing (15). Two glutathione transferases were purified from sugar borer larvae. The isozyme with a pI value of 9.3 was a homodimer of two subunits of $M_r$ 25,000, whereas the isozyme with a pI value of 8.0 was a heterodimer of subunits having $M_r$ 25,000 and 27,000. On the other hand, three glutathione transferases were purified from Mexican rice borer larvae. The isozymes with pI values of 9.7 and 7.7 were homodimers of subunits with $M_r$ 25,000 and 26,000, respectively. However, the isozyme with a pI value of 5.3 was a heterodimer of subunits having $M_r$ 26,000 and 27,000. Peptide fingerprint analysis revealed primary structural differences in these isozymes.

We have recently purified and characterized glutathione transferases from five species of Lepidoptera with differing degrees of polyphagy. The fall armyworm and corn earworm (CEW, Helicoverpa zea) are highly polyphagous insects. The tobacco budworm (TBW, Heliothis virescens) and cabbage looper are also polyphagous insects but are more specialized. The former feeds mainly on tobacco and cotton, while the latter preferentially feeds on plants in the cabbage family. The velvetbean caterpillar is a specialist insect feeding mainly on certain species of legumes.

We have found that glutathione transferases can be purified from these lepidopterous insects to apparent homogeneity using a two-step procedure involving ammonium sulfate fractionation and affinity chromatography on a glutathione-agarose column (16). Purifications were 22, 45, 37, 9 and 33-fold in the FAW, CEW, TBW, CL and VBC,

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respectively, as compared with the specific activity in the respective soluble fraction (Table III). The specific activity of the purified glutathione transferases ranged from 2.29 μmol/min/mg protein in the VBC to 58.35 μmol/min/mg protein in the CEW, a difference of 25-fold in enzyme activity.

The biochemical characteristics of purified glutathione transferases from the five lepidopterous species are shown in Table IV. Analysis of the affinity purified preparations by polyacrylamide gel electrophoresis (PAGE) under nondenaturing conditions revealed the presence of 6, 4, 1, 1 and 1 protein species in the FAW, CEW, TBW, CL and VBC, respectively. These protein species were regarded as glutathione transferase isozymes since each band catalyzed glutathione conjugation with CDNB. When the preparations were analyzed by isoelectric focusing in polyacrylamide gels, the same number of protein bands as found in PAGE are also observed in each species. The pI values ranged from 4.45 to 6.00 among the five species, indicating that these transferases were all acidic isozymes. SDS-PAGE of the purified enzymes showed 4, 3, 3, 2 and 2 protein bands in the FAW, CEW, TBW, CL and VBC, respectively. The subunit molecular weights ranged from 27,000 to 32,000 with the subunit ($M_r$ 29,000) being found in each species. Since insect glutathione transferases exist in dimers (homodimers and heterodimers) (9), the molecular weights of the native glutathione transferases would likely range from 55,000 to 64,000 based on their subunit molecular weights. It is not known why the TBW, which possessed a single form of glutathione transferase, showed three subunits in SDS-PAGE. The possibility exits that one of the subunits may have been an artifact resulting from degradation of the enzyme.

Kinetic studies showed that the purified glutathione transferases among these insects were qualitatively Ldifferent based on their $K_m$ values except for the equivalent $K_m$s of CEW and TBW. That glutathione transferases are qualitatively different in the five species was also supported by the results obtained from the enzyme inhibition study. As shown in Table IV, the TBW glutathione transferase was most sensitive to inhibition by quercetin, followed by CEW, CL, FAW and VBC.

It has recently been demonstrated for house flies that some glutathione transferase isozymes possessing DCNB activity do not necessarily bind to GSH-agarose (Clark et al., Protein Expression and Purification, in press). Therefore, the possibility of the presence of other isozymes in these lepidopterous species can not be ruled out.

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**Table III. Purification of glutathione transferases from five lepidopterous insects**[a]

| Species | Purification step | Yield (%) | Specific activity (μmol CDNB/min/mg protein)[b] | Purification factor |
|---|---|---|---|---|
| FAW | Cytosolic fraction | 100 | 0.47 ± 0.06 | 1.0 |
|  | Ammonium sulfate (45-75%) | 69 | 1.05 ± 0.13 | 2.2 |
|  | GSH-agarose | 12 | 10.37 ± 1.49 | 22.1 |
| CEW | Cytosolic fraction | 100 | 1.30 ± 0.04 | 1.0 |
|  | Ammonium sulfate | 80 | 1.93 ± 0.08 | 1.5 |
|  | GSH-agarose | 8 | 58.35 ± 2.29 | 44.9 |
| TBW | Cytosolic fraction | 100 | 0.47 ± 0.10 | 1.0 |
|  | Ammonium sulfate | 62 | 1.41 ± 0.09 | 3.0 |
|  | GSH-agarose | 10 | 17.50 ± 3.72 | 37.2 |
| CL | Cytosolic fraction | 100 | 1.35 ± 0.27 | 1.0 |
|  | Ammonium sulfate | 69 | 2.73 ± 0.47 | 2.0 |
|  | GSH-agarose | 3 | 12.16 ± 1.86 | 9.0 |
| VBC | Cytosolic fraction | 100 | 0.07 ± 0.01 | 1.0 |
|  | Ammonium sulfate | 54 | 0.11 ± 0.07 | 1.6 |
|  | GSH-agarose | 26 | 2.29 ± 0.33 | 32.9 |

[a] Adapted from ref. 16.
[b] Mean ± SE.

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**Table IV. Biochemical characteristics of purified glutathione transferases from five lepidopterous insects**[a]

| Species | $K_m$ (mM) | $V_{max}$ (μmol/min/ mg protein) | Inhibition by quercetin (% of control) | | Subunit $M_r$ | Isozyme ($R_f$) | pI |
|---|---|---|---|---|---|---|---|
| | | | $10^{-4}$ M | $10^{-5}$ M | | | |
| FAW | 1.33 | 17.2 | 23 | 71 | 30,000 | 0.34 | 6.0 |
| | | | | | 29,500 | 0.42 | 5.10 |
| | | | | | 29,000 | 0.46 | 5.00 |
| | | | | | 28,200 | 0.51 | 4.75 |
| | | | | | | 0.55 | 4.55 |
| | | | | | | 0.59 | 4.45 |
| CEW | 0.40 | 50.0 | 0 | 12 | 32,000 | 0.38 | 5.10 |
| | | | | | 29,000 | 0.42 | 5.05 |
| | | | | | 27,500 | 0.46 | 4.90 |
| | | | | | | 0.54 | 4.75 |
| TBW | 0.40 | 50.0 | 0 | 0 | 32,000 | 0.47 | 5.45 |
| | | | | | 29,000 | | |
| | | | | | 27,500 | | |
| CL | 0.56 | 12.5 | 22 | 46 | 31,000 | 0.53 | 5.10 |
| VBC | 9.09 | 18.2 | 100 | 100 | 31,000 | 0.44 | 4.85 |
| | | | | | 29,000 | | |

[a] Affinity purified preparation from each species was used as enzyme source toward CDNB.

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In Molecular Mechanisms of Insecticide Resistance; Mullin, C., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1992.

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## Induction of Glutathione Transferases by Allelochemicals in Lepidoptera

Glutathione transferases are induced by xenobiotics such as barbiturates and pesticides in mammals (17) and in insects. Ottea and Plapp (18) found that glutathione transferase was induced nearly 3-fold in house flies by dietary phenobarbital. Hayaoka and Dauterman (19) also obtained induction in house flies by various dietary insecticides with chlorinated hydrocarbons being most active. More recently, Capua et al. (20) have demonstrated induction of glutathione transferase in the bulb mite (Rhizoglyphus robini) by numerous xenobiotics, including pentobarbital and the insecticides fenpropathrin and propoxur.

Glutathione transferases in lepidopterous insects are induced by numerous host plants. Umbellifers and crucifers were better inducers of these enzymes than other plants screened (Table V). Parsnip was the best inducer among those tested, causing a 39-fold increase compared with activity in larvae fed artificial diet. However, plants such as soybeans, sorghum, millet, Bermudagrass, corn, potato, cucumber, carrot and broccoli had no effect on this enzyme (21,22). Time course studies showed that the maximum induction of the transferase by cowpeas occurred two days after feeding began (21). The methanol leaf extract of a resistant soybean variety (PI227687) induced glutathione transferase in the soybean looper (Pseudoplusia includens) (23).

Induction of glutathione transferases also occurs in deciduous tree-feeding insects. Lindroth (24) found that glutathione transferase activities in the luna moth (Actias luna) larvae fed black walnut, butternut and shagbark hickory were 2 to 3-fold higher than in those fed paper birch. Furthermore, microsomal glutathione transferase activity varied up to 5-fold among eastern tiger swallowtail (Papilio glaucus glaucus) larvae fed black cherry, tulip tree, paper birch, white ash and basswood (25).

The induction is believed to be due to allelochemicals in the plants. The identity of the glutathione transferase inducer in parsnip leaves was determined by thin-layer chromatography, high-pressure liquid chromatography, gas chromatography, and mass spectrometry as xanthotoxin, a linear furanocoumarin (26). Other allelochemicals such as indole 3-acetonitrile, indole 3-carbinol, indole 3-ß-D glucoside, flavone, allyl isothiocyanate, benzyl isothiocyanate, 2-phenylethyl isothiocyanate, benzyl thiocyanate and sinigrin also induced the transferase in fall armyworms (Table V). Xanthotoxin also induced glutathione transferase in the black swallowtail (Papilio polyxenes) and cabbage looper and harmine, an indole analog, induced the transferase in the cabbage looper (27).

In addition, dietary coumarin and monoterpenes (α-pinene, ß-pinene, limonene, terpinene) induced glutathione transferase in southern armyworm (Spodoptera eridania) larvae (28). However, monoterpenes were not inducers of the transferase in fall armyworm larvae (21). Coumestrol, a coumarin analog found in a resistant soybean cultivar, induced glutathione transferase in soybean loopers (29). 2-Tridecanone found in wild tomato leaves induced glutathione transferase in tobacco budworm larvae (30).

Kinetic study revealed a quantitative, but no qualitative difference between the glutathione transferase of soybean- and cowpea-fed fall armyworms (21). These results support the notion that these induced transferases are not separate isozymes. Our recent work also indicated that induction of glutathione transferase in fall armyworm larvae by xanthotoxin increased levels of the existing isozymes but did not result in production of a new isozyme (16).

Glutathione transferases toward the toxic allelochemicals trans-cinnamaldehyde, benzaldehyde, allyl isothiocyanate, benzyl isothiocyanate and benzyl thiocyanate can be induced by various allelochemicals including respective substrates (Table VI). Hence allelochemical induction of its own metabolism can occur in phytophagous insects. However, xanthotoxin, a potent inducer of the enzyme, was not metabolized by glutathione transferase from larvae of the fall armyworm (6) and black swallowtail (27).

Using two allelochemicals, xanthotoxin and indole 3-acetonitrile, as inducers of glutathione transferase (toward DCNB), marginal induction (16-39%) was found in the specialist VBC compared to 1580-2544% in the generalist FAW (Figure 1). Enzyme assays in individual larvae showed that inducibility of glutathione transferase varied considerably in the populations of the specialist and generalist species (Figures 2 and 3). All individuals were induced in the FAW population compared to only one third of the VBC population being induced. Moreover, the net increase in enzyme activity due to induction was much higher in the FAW than in the VBC. The observed differences in enzyme inducibility could be attributed to the qualitative as well as quantitative differences in glutathione transferase isozymes in these species.

## Role of Glutathione Transferases in Insect Herbivory

As mentioned above, glutathione transferases are involved in the in vitro metabolism of toxic allelochemicals in phytophagous Lepidoptera. The allelochemicals trans-cinnamaldehyde, trans-hexenal, trans,trans-2,4-decadienal and benzaldehyde, all of which contain the α,ß-unsaturated carbonyl moiety, were metabolized by glutathione transferase from midgut homogenates of FAW.

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**Table V. Glutathione transferase activity of fall armyworm larvae fed host plants and allelochemicals[a]**

| Inducer | Glutathione transferase (DCNB) (% of artificial diet-fed insects) |
|---|---|
| Cotton | 142 |
| Peanuts | 179 |
| Chinese cabbage | 221 |
| Cabbage | 404 |
| Collards | 472 |
| Cowpeas | 493 |
| Radish | 636 |
| Turnip | 731 |
| Mustard | 782 |
| Parsley | 1944 |
| Parsnip | 3909 |
| Indole 3-acetonitrile (0.2%) | 1801 |
| Indole 3-carbinol (0.2%) | 388 |
| Indole 3-ß-D-glucoside (0.2%) | 202 |
| Flavone (0.2%) | 687 |
| Sinigrin (0.2%) | 342 |
| 2-Phenylethyl isothiocyanate (0.025%) | 217 |
| Xanthotoxin (0.01%) | 2644 |

[a] Adapted from refs. 21, 22 and 26.

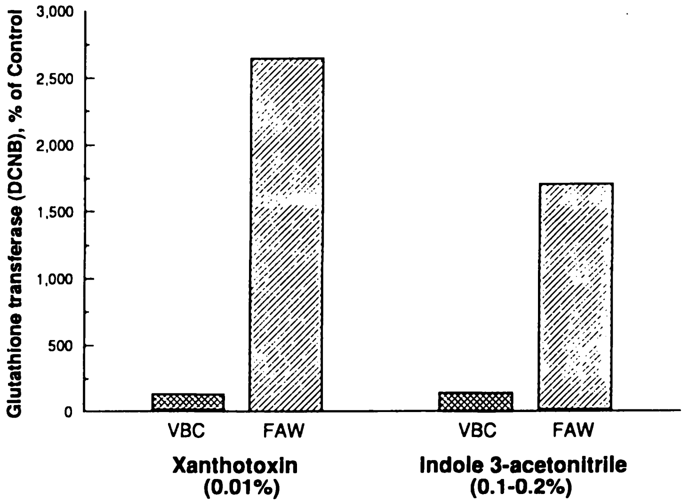

Fig. 1. Induction of glutathione transferase activity by allelochemicals in velvetbean caterpillar and fall armyworm larvae.

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**Table VI. Effect of dietary allelochemicals on glutathione transferase activity toward allelochemicals in fall armyworm larvae[a]**

| Treatment[b] | Glutathione transferase with substrate (% of control activity) | | | | |
|---|---|---|---|---|---|
| | *Trans*-cinnamaldehyde | Benzaldehyde | Allyl isothiocyanate | Benzyl isothiocyanate | Benzyl thiocyanate |
| Indole 3-acetonitrile | 218 | 249 | 1092 | 550 | 515 |
| Indole 3-carbinol | | | 550 | | 412 |
| Flavone | 217 | | 625 | | 426 |
| Xanthotoxin (0.01%) | | | 558 | | 456 |
| *Trans*-cinnamaldehyde | 93 | | | | |
| Benzaldehyde | | 237 | | | |
| Allyl isothiocyanate (0.025%) | | | 542 | | |
| Benzyl isothiocyanate (0.025%) | | | | 1006 | |
| Benzyl thiocyanate | | | | | 162 |

[a] Adapted from refs. 6-8.

[b] Newly molted sixth-instar larvae were fed artificial diets containing the allelochemicals (0.2%, unless otherwise stated) for two days before enzyme assays were conducted.

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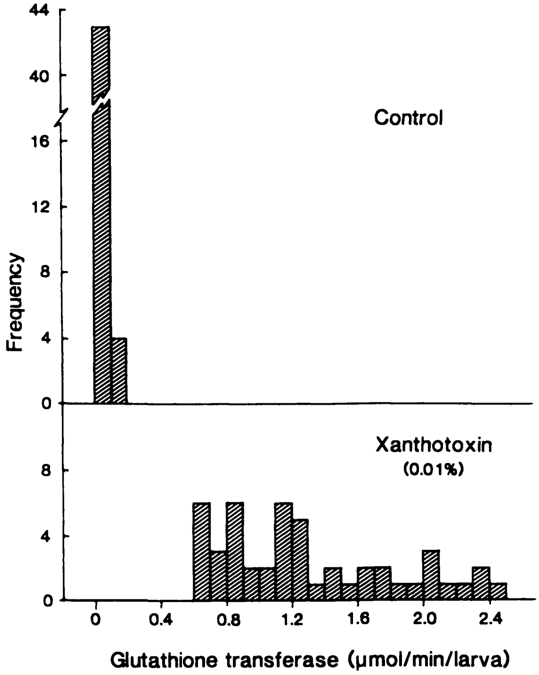

Fig. 2. Histograms for glutathione transferase activity (DCNB) in individual larvae of the fall armyworm fed xanthotoxin (0.01%) (Yu, S. J. J. Econ. Entomol., in press).

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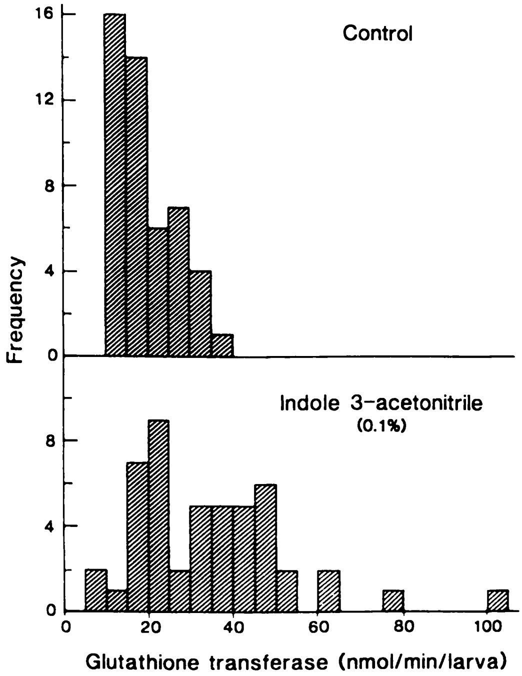

Fig. 3. Histograms for glutathione transferase activity (DCNB) in individual larvae of the velvetbean caterpillar fed indole 3-acetonitrile (0.1%).

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Isothiocyanates such as allyl, benzyl and 2-phenylethyl isothiocyanates and organothiocyanates such as benzyl thiocyanate were metabolized by glutathione transferase from larvae of the FAW, CL and VBC. Furthermore, numerous allelochemicals induced glutathione transferase activity which is presumed to enhance the detoxication of allelochemicals. In addition, glutathione transferase activity toward various allelochemicals was lower in the specialist VBC than that in the generalists FAW and CL, and activity toward the isothiocyanates in the crucifer-adapted cabbage looper was 2- to 6-fold higher than that in the fall armyworm. The results support the notion that glutathione transferases play an important role in the feeding strategies of lepidopterous insects. The highly polyphagous insects may have evolved multiple glutathione transferases to detoxify the diverse toxic allelochemicals encountered in their host plants. On the other hand, specialized insects which feed on a narrow range of host plants and encounter more specific allelochemicals have as few as one form of glutathione transferase. Therefore, the isozyme composition of glutathione transferase in lepidopterous species is related to host plant feeding. More work is needed to learn the substrate specificities of these isozymes before one can fully understand the molecular mechanisms of glutathione-dependent detoxication in these Lepidoptera. These results suggest that glutathione transferases play an important role in allelochemical resistance in phytophagous Lepidoptera.

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## Chapter 15

# Mechanism of Insect Resistance to *Bacillus thuringiensis* in *Plodia interpunctella* and *Plutella xylostella*

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The mechanism of resistance to *Bacillus thuringiensis* insecticidal crystal proteins (ICPs) was investigated in a laboratory-selected and a field-selected insect strain. In both cases, insect resistance appeared to be due to changes in ICP receptors, suggesting that alterations in ICP receptors are a general mechanism by which insects can adapt to *B. thuringiensis*. The absence of cross resistance to ICPs other than those present in the selecting agent and the finding that these ICPs bind to distinct receptors indicate that the use of ICP mixtures or multiple ICP expressing transgenic plants may be a valuable resistance management tactic.

## Insect Control with *Bacillus thuringiensis*

Contemporary agriculture heavily relies on chemical inputs such as pesticides and fertilizers. Worldwide, approximately 6000 million dollars (US) is spent yearly on insecticides alone. Increasing costs for developing new compounds, public concern about the environmental impact of pesticides and the problem of insect resistance have stimulated interest in alternative insect control agents. Products based on the bacterium *Bacillus thuringiensis* are the most widely used biopesticides.

The insecticidal activity of *B. thuringiensis* is due predominantly to a proteinaceous crystalline inclusion, produced during sporulation, and consisting of one or more proteins called delta- endotoxins or insecticidal crystal proteins (ICPs). In the insect midgut, crystals are dissolved and the liberated ICPs or protoxins are proteolytically processed to a toxic fragment of about 60 kDa. This fragment (the toxin) binds to specific receptors on the epithelium of midgut cells, resulting in perturbation of the ionic balance and eventually lysis of these cells. A considerable number of distinct ICP types have been characterized, each having a unique insecticidal spectrum *(1)*.

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In Molecular Mechanisms of Insecticide Resistance; Mullin, C., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1992.

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Fifty years after it was isolated, *B. thuringiensis* was developed as a microbial insecticide. Today, a considerable number of *B. thuringiensis* based products are commercially available *(2)*. The majority of the *B. thuringiensis* varieties and commercial products are active against lepidopteran insects. The discovery and rapid commercialization of dipteran *(3)* and coleopteran *(4)* specific strains expanded the market potential of *B. thuringiensis* considerably. As a result, *B. thuringiensis* is now used to control lepidopteran and coleopteran pests in both agriculture and forestry and to eradicate dipteran disease vectors. In addition to conventional applications, recent advances in transformation technology made it possible to express cloned ICP genes in plants, thereby overcoming some of the disadvantages of *B. thuringiensis* sprays *(5-8)*.

*B. thuringiensis* preparations are harmless for non-target insects, vertebrates, the environment and the user because of the highly specific action of ICPs *(9)*. Accordingly, *B. thuringiensis* can be applied until the day of harvest. Thus, this microbial insecticide appears to be an environmentally sound alternative for synthetic insect control agents.

## Insects Can Adapt to *B. thuringiensis*

Obviously, the extent to which insects are able to develop resistance to ICPs will be an essential determinant for the continued success of *B. thuringiensis* based insect control. Laboratory selection experiments with *B. thuringiensis* ICPs have been performed with at least 8 different insect species. In most studies, no or very low levels of resistance were obtained, leading to the presumption that resistance to *B. thuringiensis* would be unlikely *(10)*. However, recent selection experiments have shown that insects do have the potential to develop significant resistance against *B. thuringiensis*. A *Plodia interpunctella* strain, reared on a diet containing Dipel, a commercial formulation of a spore/crystal mixture from *B. thuringiensis* subsp. kurstaki HD1, developed a 30-fold resistance level in only two generations *(11)*. Resistance levels as high as 250-fold were observed after 36 generations. Subsequently, lower levels of resistance have been selected in the almond moth (*Cadra cautella*) *(12)* and the tobacco budworm (*Heliothis virescens) (13)*.

Still, many believed (or hoped?) that resistance development in laboratory selection experiments would be artifactual and not related to the potential for the emergence of resistant insect populations in the field. In 1988 Kirsch and Schmutterer reported low efficacy of *B. thuringiensis* in the control of *P. xylostella* in the cabbage growing area around Baguio City in the Philippines *(14)*. It was suggested that the insects had developed some degree of resistance to *B. thuringiensis*, although alternative explanations may be possible. The first case where a reduced effectiveness of *B. thuringiensis* insect control could be attributed to resistance of insect populations was reported by Tabashnik et al *(15)*. One population of *P. xylostella* isolated from fields treated with *B. thuringiensis* was 25 to 33 times less susceptible than two unchallenged laboratory colonies. Field rate doses gave 90 to 100 % mortality in these latter colonies but only maximally 35 % mortality in two resistant populations. Field resistance has also been observed in another *P. xylostella* population in an area repeatedly exposed to Dipel *(16)*.

Although transgenic plants producing their own protective proteins (ICPs) provide an exciting new approach to insect control, the concern has been voiced that a large scale introduction of crops containing a monogenic resistance trait could rapidly lead to the development of resistance within insect populations. Moreover, the greater residual presence of the ICPs in transgenic plants as compared to sprays could increase selection pressure and the risk of resistance.

To warrant continued success of *B. thuringiensis* we should anticipate resistance problems and develop strategies to eliminate or decrease the resistance potential. Therefore, it is critical to understand the mechanism involved in pest resistance to these toxins.

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## Mechanism of Insect Resistance to *B. thuringiensis* ICPs

Insect resistance may be due to several etiological, physiological or biochemical phenomena, including a behavioral change, a decrease in uptake of the insecticide, sequestration, accelerated excretion, metabolic detoxification of the toxic compound or target site insensitivity. As we have demonstrated a correlation between toxicity and binding for several ICP - insect combinations *(17-19)*, we hypothesized that resistance against *B. thuringiensis* could be due to altered membrane binding of toxin.

First, we investigated the mechanism of resistance to *B.thuringiensis* ICPs in a *Plodia interpunctella* strain selected for a high level of resistance against Dipel *(11)*. We have compared resistance levels to Dipel and to trypsin activated CryIA(b) and CryIC ICP *(20)*. It should be noted here that crystals of Dipel contain ICPs belonging to the CryIA and CryII family, but no CryIC ICPs. Table I summarizes the toxicity data for the sensitive strain (S strain) and for the strain selected for Dipel resistance (R strain). High levels of resistance were observed for Dipel and CryIA(b). In contrast, there was no resistance to CryIC. Moreover, we observed a marked increase in sensitivity of the R strain to CryIC.

Table I. Toxicity of *B. thuringiensis* ICPs to sensitive and resistant *Plodia interpunctella* larvae

| ICP | $LD_{50}$ (μg/larva) | |
|---|---|---|
| | S strain | R strain |
| Dipel | 1.21 (0.88-1.84) | > 30 |
| CryIA(b) | 0.03 (0.02-0.05) | 26.3 |
| CryIC | 0.11 (0.08-0.15) | 0.03 (0.00-0.07) |

SOURCE: Adapted from ref. 20.

In order to investigate whether altered membrane binding would be responsible for resistance, we performed receptor binding studies with $^{125}$I- labeled CryIA(b) and CryIC and brush border membrane vesicles derived from larval midguts of the R and S strains (Table II) *(20)*. From homologous competition experiments, a dissociation constant ($K_d$) of 0.72 ± 0.26 nM and a binding site concentration ($R_t$) of 1.44 ± 0.35 pmoles/mg membrane protein was calculated for binding of CryIA(b) to vesicles of the S strain. A 50 fold reduction in binding affinity ($K_d$ = 36.3 ± 22.7 nM) was determined in the R strain. The binding site concentration remained virtually unchanged ($R_t$ = 1.77 ± 0.58 pmoles/mg membrane protein).

Binding studies were also performed with CryIC. In the S strain, both high and low affinity binding sites were present. In the R strain, only high affinity binding of CryIC was detected, although with different $K_d$ and $R_t$ values. Whereas the difference in $K_d$ for the high affinity site in the two strains was only marginally significant, the increase in $R_t$ in the R strain is statistically significant. Thus, in parallel with the higher sensitivity of the R strain to CryIC as compared to the S strain, the binding of this toxin was increased in the R strain.

Table II. Binding characteristics of *B. thuringiensis* ICPs on brush border membrane vesicles from larval midguts of sensitive and resistant *Plodia interpunctella* larvae

| ICP | Strain | $K_{d1}$ (nM) | $R_{t1}$ (pmol/mg protein) | $K_{d2}$ (nM) | $R_{t2}$ (pmol/mg protein) |
|---|---|---|---|---|---|
| | | (high affinity | site) | (low affinity | site) |
| CryIA(b) | S | 0.72 (±0.26) | 1.44 (±0.35) | | |
| | R | 36.3 (±22.7) | 1.77 (±0.58) | | |
| CryIC | S | 0.31 (±0.12) | 0.38 (±0.07) | 154 (±108) | 6.17 (±3.79) |
| | R | 0.18 (±0.08) | 1.15 (±0.20) | | |

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Results from heterologous competition experiments suggested that the low affinity site for CryIC in the S strain represents the CryIA(b) binding site *(20)*. We assume that this site, which in the R strain exhibits a 50 fold reduction in affinity for CryIA(b), would also display a reduced affinity for the CryIC. This would result in a very low affinity, explaining why a second site for CryIC is not detected in the R strain.

Secondly, we investigated the resistance mechanism in a *P. xylostella* population that had developed resistance in response to foliar applications of Dipel in the field *(16)*. Such a study appeared to be very relevant since laboratory- and field-selected resistance may be due to different factors *(21)*. We studied toxicity and binding of three distinct trypsin activated ICPs, CryIA(b), CryIB and CryIC, to

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the R strain and S strain *(16)*. Dipel crystals contain CryIA ICPs, but no CryIB or CryIC ICPs. All three ICPs were toxic to the S strain, although to different extents (Table III). The R strain was as susceptible to CryIB and CryIC as the S strain, but about 200 times less susceptible to CryIA(b).

Table III. Toxicity of *B. thuringiensis* ICPs to sensitive and resistant *Plutella xylostella* larvae

| ICP | $LC_{50}$(μg/larva) | |
|---|---|---|
| | S strain | R strain |
| CryIA(b) | 6.7 (2.8-16.1) | >1350 |
| CryIB | 1.2 (0.8-1.7) | 2.3 (0.7-5.3) |
| CryIC | 88.9 (43.8-164.6) | 46.5 (23.9-84.4) |

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Binding studies using the three ICP types and increasing concentrations of brush border membrane vesicles from both strains revealed a virtually identical binding pattern on the two strains for CryIB and CryIC. However, while a considerable level of specific binding was observed between CryIA(b) and vesicles from the S strain, no significant binding was detected in the R strain (Figure 1). In order to obtain quantitative data of the binding characteristics of the ICPs under study, homologous competition experiments were performed (Table IV). A $K_d$ value of 4.2 ± 2.5 nM and an $R_t$ value of 1.6 ± 1.2 pmol/mg vesicle protein was calculated for binding of CryIA(b) to the S strain. No values for binding to the R strain could be obtained because of the virtually complete lack of specific binding. Apparently, the R strain had lost the capacity to bind CryIA(b). The altered membrane binding of this ICP may be due to a decrease in affinity or concentration of the CryIA(b) receptor, or both. Binding characteristics of CryIC were not significantly different between the two strains. We were not able to determine the $K_d$ and $R_t$ values for CryIB due to the low specific radioactivity of labeled preparations of this ICP type.

Heterologous competition experiments indicated that the binding site for CryIC is not recognized by CryIA(b) or CryIB *(16)*. The binding sites for the latter two ICP types are probably also distinct sites since binding of CryIA(b) is dramatically different in both strains but binding of CryIB is virtually identical in the two strains. We concluded that the laboratory strain has (at least) three distinct ICP receptors, for CryIA(b), CryIB and CryIC, respectively. The resistant strain has either lost CryIA(b) receptors or possesses receptors defective in binding CryIA(b).

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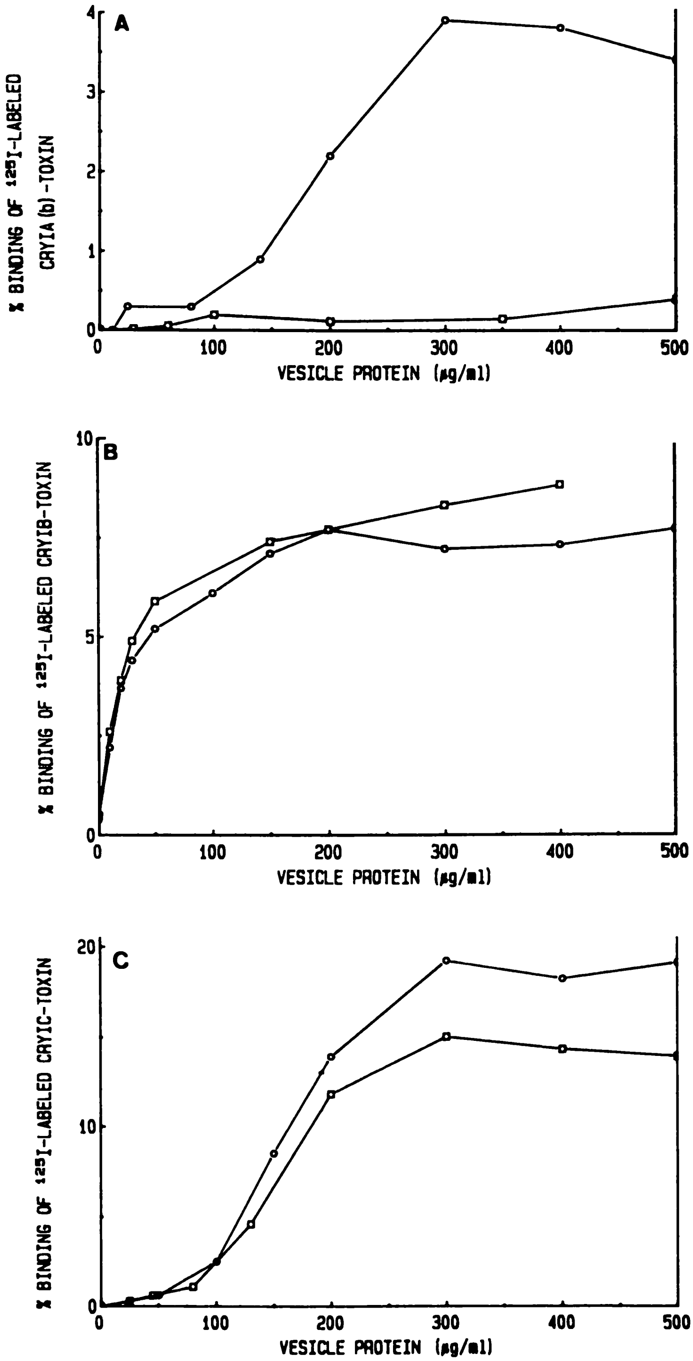

Figure 1. Binding of $^{125}$I-labeled CryIA(b) (A), CryIB (B) and CryIC (C) to brush border membranes from sensitive (open circles) and resistant (open squares) *P. xylostella* larvae. (Reproduced with permission from ref. 16. Copyright 1991 J. Ferré.)

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Table IV. Binding characteristics of *B. thuringiensis* ICPs on brush border membrane vesicles from larval midguts of sensitive and resistant *Plutella xylostella* larvae

| ICP | Strain | $K_d$ (nM) | $R_t$ (pmol/mg protein |
|---|---|---|---|
| CryIA(b) | S | 4.2 (± 2.5) | 1.6 (± 1.2) |
| | R | N.D.[a] | N.D.[a] |
| CryIC | S | 6.5 (± 1.5) | 10.8 (± 7.3) |
| | R | 7.6 (± 2.0) | 2.9 (± 0.3) |

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[a] N.D.: not determined.

In conclusion, we have shown in two insect strains that resistance against *B. thuringiensis* is due to a change in ICP membrane receptors. Resistance of the laboratory-selected *P. interpunctella* strain to CryIA(b) was correlated with a reduced affinity of the CryIA(b) receptor site. The increased sensitivity of this strain to CryIC was reflected in an increased CryIC binding site concentration. Field-selected resistance in a *P. xylostella* strain appeared to be due to loss of (functional) CryIA(b) receptors. The observation that the biochemical mechanism responsible for resistance is independent of the background of selection indicates that alterations in ICP receptors is probably a general mechanism by which insects can adapt to *B. thuringiensis.*

## Implications for Resistance Management

Our results suggest that the use of *B. thuringiensis* formulations containing different ICPs which bind to different receptors or transgenic plants expressing multiple ICPs may be a valuable strategy to delay resistance. It is indeed generally accepted that resistance buildup will be delayed by using mixtures of insecticides having different target sites *(22)*. Some experimental results provide evidence for the validity of this proposal. Laboratory selection experiments with *B. thuringiensis* subsp. israelensis on *Culex quinquefasciatus* resulted in low levels of resistance *(10)*. However, when after 92 generations of selection with the complex of *B. thuringiensis* subsp. israelensis ICPs, a subcolony was subjected to selection by a single *B. thuringiensis* subsp. israelensis ICP, a considerable level of resistance evolved relatively rapidly *(10)*. Selection experiments with a *Pseudomonas fluorescens* strain, engineered to produce CryIA(c) ICP, on *H. virescens* resulted in a 24-fold resistance level after 7 generations. However, only a 3.8-fold resistance level was observed to Dipel, containing a mixture of CryIA and CryII ICPs and spores *(13)*. Also, although we observed a more than 200-fold resistance level to CryIA(b) in a field- selected *P. xylostella* population, we could not demonstrate significant resistance to Dipel *(16)*.

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In whatever way *B. thuringiensis* is employed, it is probably wise to keep selection pressure as low as possible. In the case of transgenic crops, restriction of ICP expression to those plant tissues which are most susceptible to pest damage could decrease selection pressure, while still providing adequate protection. Another option might be to provide refuges in a temporal fashion (by activating ICP gene expression only during a particular period of the growth season) or a spacial fashion (by mixing susceptible with resistant plants) *(23)*. A different approach consists in the development of plants with wound-induced ICP expression. Transgenic plants with fine-tuned ICP expression could become part of integrated pest management programs, in which intense pest control measures are only taken in reference to economic damage thresholds *(24)*.

Currently available data on insect resistance in general and resistance to *B. thuringiensis* in particular suggest we should use *B. thuringiensis* intelligently. If we do not, the usefulness of this environmentally safe insecticide may be lost due to pest adaptation.

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## Chapter 16

# *Tribolium* as a Model Insect for Study of Resistance Mechanisms

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Classical techniques for chromosome manipulation at the organismal level are used routinely by *Drosophila* biologists to facilitate the study of genetic variation in populations, but such techniques are lacking in other insects. We are attempting to develop the genetic tools needed to carry out such manipulations in the beetle *Tribolium castaneum*, and to apply them to the study of several types of biological problems, including insecticide resistance. In principle, artificial mutagenesis can be used to induce resistance mutations at a frequency much higher than the spontaneous rate, providing an efficient way to identify genes capable of conferring resistance. Balancer chromosomes, carrying crossover-suppressing rearrangements, lethals, and dominant visible markers, can be used to extract and render homozygous such mutations, whether recessive or dominant. We have demonstrated the feasibility of applying this method to approach the saturation mutagenesis of a portion of the second linkage group, and are now applying it to a search for pathogen resistance mutations.

Only a limited number of eukaryotic species are sufficiently tractable as genetic models to qualify as preferred experimental subjects in which a broad range of sophisticated molecular and genetic techniques can be employed for biological research. Any species that would aspire to such preferred status should possess at least a few of the following properties: 1. A short generation time and ease of rearing, handling and making genetic crosses; 2. A genome small in size, well mapped by visible and molecular markers, and containing only minimal amounts of highly dispersed repetitive DNA; 3. A capacity for *in vivo* chromosome manipulations using deletions, duplications and balancer chromosomes to facilitate genetic mapping, dosage analysis, reversion analysis and chromosome extraction (described in greater detail below); and 4. A capacity for germline transformation and transposon-mediated gene tagging and cloning.

Among higher animals, the insect *Drosophila melanogaster* provides the most powerful experimental system for integrated genetic and molecular studies. This organism was first chosen for property #1 from the above list, but its potential wasn't fully realized until techniques were developed to exploit properties #3 and 4, aided by #2. Today, the utility of *Drosophila* as an animal model system is rivalled only by the

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nematode *Caenorhabditis elegans*, for which the experimental advantages of inbreeding have been exploited rather than chromosome manipulation.

Although the experimental advantages of *Drosophila* make it the preferred species for detailed examination of the molecular and genetic mechanisms of insecticide resistance, other insect models are clearly needed. In view of the great diversity of insect forms and the unique importance of insects to mankind, much effort is now being devoted to mapping the genomes of several economically important insect species with molecular markers, and to developing new techniques for germline transformation. The absence of methods for chromosome manipulation and other sophisticated genetic techniques in these species places limits on the kinds of information and strategies possible. We have recently shown that the beetle *Tribolium castaneum* has many of the characteristics that have proven so favorable in *Drosophila* research. Below, we describe the attributes of *Tribolium* that recommend it as a genetic model. We further describe our initial efforts to develop the chromosome extraction technique in this insect, and discuss our progress toward incorporating the technique into a genetic analysis of pathogen resistance.

## *Tribolium castaneum* (Red Flour Beetle)

The red flour beetle (hereafter referred to as RFB) is globally one of the most abundant and widespread pests infesting stored grain, flour and other cereal products. Its ease of laboratory culture has made it a popular system for studies in population dynamics, population genetics and quantitative genetics for over 30 years. It has by far the best-marked set of genetic linkage maps in the Coleoptera, and one of the best among all insects. It is one of the easiest of all insects to rear and handle in large numbers, can readily be mutagenized, and is amenable to high resolution genetic analysis (*1*). A compilation of information on the genetics and biology of the genus *Tribolium* is given in a three volume set (*2-4*), and in an annual newsletter entitled the *Tribolium* Information Bulletin, containing stocklists, research results and bibliographies, .

RFB is also particularly well suited for molecular genetic study. It has a genome size of 0.21 pg per haploid genome (*5, 6*), comparable to that of *Drosophila melanogaster*, making it one of the smaller insect genomes known. The RFB genome, like that of *D. melanogaster*, has a long-period interspersion pattern of repetitive elements, in which the moderaterly repetitive sequences are well-separated by long tracts of unique-sequence DNA. The RFB genome is organized into a relatively small number of chromosomes: nine pairs of autosomes and one pair of sex chromosomes (n=10). Sex is determined by a simple XX, XY system in which males are the heterogametic sex. This karyotype is typical of many beetle species (*7*). A diploid chromosome number and meiotic recombination occur in both sexes.

RFB has shown great adaptability in developing resistance to all classes of insecticides to which it has been exposed, including pyrethroids (*8*), organophosphates (*9*), DDT (*10*), juvenile hormone analogues (*11*) and fumigants (*12*). The practicality of genetically extracting, purifying and mapping genes for insecticide resistance, including genes for carboxylesterase-related malathion resistance and target insensitivity-type cyclodiene resistance, has also been demonstrated in RFB (*13-15*).

In short, the economic significance of RFB, its favorable biological and genetic attributes and its long history of insecticide resistance strongly recommend this species as a model genetic system for the study of insecticide resistance mechanisms. Our efforts are currently focussed on constructing balancer chromosomes and other rearrangements which will facilitate chromosome manipulations; mapping the genome with visible genetic markers, recessive lethal mutations and restriction fragment length polymorphisms (RFLPs); developing a transposon-mediated gene tagging and transformation system; and inducing and characterizing insecticide and pathogen resistance mutations.

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## Chromosome Extraction: General Principles

Among the most powerful genetic tools in Drosophila research are multiply-rearranged "balancer" chromosomes used to maintain the integrity of homologs which have been mutagenized or sampled from wild populations. Such balancers typically carry multiple inversions which decrease or totally eliminate the recovery of viable recombinant progeny, as well as a dominant visible marker and a recessive lethal to aid in following the transmission of the balancer and in eliminating homozygous progeny, respectively. Indeed, the utilization of such a balancer for the X chromosome was the key to Muller's Nobel Prize-winning work on the induction of mutations, performed more than six decades ago. Incredibly, this valuable research tool has not been systematically utilized for any higher animal outside of the Drosophilids.

Extraction of mutagenized chromosomes is in principle a powerful strategy for discovering previously unknown resistance loci. In *Drosophila*, this approach has been used to identify methoprene target resistance, previously unknown in any insect species. In this case the discovery had important implication for basic studies on the mode of action of juvenile hormone (JH) analogs and of JH itself (T. G. Wilson, this volume). The availability of crossover-suppressing rearrangements, recessive lethals and dominant visible mutations in the RFB has suggested the possibility of chromosome extraction in this species.

The principle by which chromosome extraction operates is illustrated in Figure 1. It is a three-step process involving extracting, amplifying and rendering homozygous the desired chromosome. Two types of marked homologs of the chromosome to be extracted are required. The first is a balancer chromosome carrying a crossover suppressor =C, a lethal =x, and a dominant visible marker =D1. The second type must carry a dominant visible =D2, different from the one on the balancer chromosome, and must have no lethals in common with the balancer chromosome. The crossover suppressor C allows retention of the identity and integrity of the original chromosome during extraction, amplification and generation of homozygotes by preventing recombination (i.e. contamination) with homologous chromosomes during meiosis. The dominant visible marker D2 allows recognition of individuals carrying both the extracted chromosome and the balancer chromosome after the amplification cross, since such individuals possess the D1 phenotype but lack the D2 phenotype. The lethal x prevents the development of D1 homozygotes which would be indistinguishable from the desired class. Finally, the D1 marker allows recognition of individuals homozygous for the desired chromosome after the final cross, since these individuals will lack the D1 phenotype. By this method all progeny from cross #3 (Figure 1C) that are phenotypically normal with respect to D1 and D2 must be homozygous for the extracted chromosome, i.e. the method is 100% efficient. Other regions of the genome can be extracted at reduced efficiency by inbreeding in cross #3, that is by using the same $F_1$ male as sire for crosses 2-3 (Figure 1B-C) so that a single $F_1$ male is crossed to his daughters for the final homozygote-generating step.

## Chromosome Extraction in *Tribolium*

In our initial feasibility study for chromosome extraction in RFB we used the two homologous chromosomes, *maxillopedia-Dachs*$^3$ (*mxp*$^{Dch3}$) and *Eyeless (Ey)*. These chromosomes represent the second linkage group (LG2), and emerged from our studies of a cluster of homeotic genes located on this linkage group *(1, 16-18)*. The *mxp*$^{Dch3}$ chromosome represents the first type (balancer) described above, and contains C, D1 and x traits, while the *Ey* chromosome corresponds to D2. *mxp*$^{Dch3}$ is a single, radiation-induced mutation that confers all three necessary properties C, D1

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and x. That is, *mxp*$^{Dch3}$ is a crossover suppressor; has a dominant visible phenotype (=short legs); and is lethal when homozygous. The *Ey* chromosome has a dominant phenotype (=reduction in the number of ommatidia in the compound eyes) different from that on the *mxp*$^{Dch3}$ chromosome, and lacks any lethal present on the *mxp*$^{Dch3}$ chromosome (i.e. *mxp*$^{Dch3}$ */Ey* is a viable genotype), although the *Ey* chromosome does carry an unrelated lethal that is balanced by *mxp*$^{Dch3}$.

Ideally, a balancer should balance an entire chromosome, i.e. it should eliminate all recombination over the length of the chromosome. The extent of the region of LG2 balanced by *mxp*$^{Dch3}$ is uncertain, but appears to be approximately in the range of 20-50 map units (ref. *19* and unpublished observations) which may correspond to one chromosome arm. The total recombinational length of the RFB genome has been estimated at ca. 1000 map units (unpublished observations), or about fourfold greater than that of *Drosophila*. The karyotype of LG2 is unknown.

After ethylmethanesulfonate (EMS) mutagenesis and extraction of LG2, we screened for lethals and for random visible mutations. The results are summarized in Table I. Among 1607 chromosomes screened we found 15 independently-derived lethals representing 7 complementation groups. Many other lethals were found, but because they occurred in the same batches as the aforementioned, they could not be confirmed to be independently-derived. Complementation analysis has not been completed for those in this latter category. In addition, we found 7 independently-derived visible mutations causing a variety of unique and distinct morphological or behavioral abnormalities. These include the two behavioral abnormalities "*tremorous*" and "*prolapsed genitalia*", and the morphological abnormalities "*wingless*", "*short elytra*", "*broken antennae*", "*epidermal hypertrophy*" and "*melanized quinone gland*". Detailed genetic analysis of these mutations will be published elsewhere. Assuming that the genome has 5000 genes and that *mxp*$^{Dch3}$ balances 2-5% of the genome, then 100-250 genes are potentially identifiable by mutation, whereas a minimum of 14 genes were in fact identified. The true extent of the balanced region is uncertain.

## Chromosome Extraction and Pathogen Resistance Genes

Having demonstrated that chromosome extraction is feasible in RFB, we can now envision the application of this technique to the search for genes that control resistance to pathogens (or other insecticides) in this species. The mutagenesis and extraction procedure would be unchanged, but instead of screening for visible or lethal mutations, we would screen for variants that can survive discriminating doses of *Bacillus thuringiensis* var. *tenebrionis* (BT).

An advantage of this approach is that recessive resistance mutations will be detected. Indeed, the first case of field derived pathogen resistance ever reported in an insect involved recessive resistance to BT *(20)*. Under laboratory or field conditions recessive resistance ordinarily has a lower probability of being selected than dominant resistance, even if the two occur with equal frequency. The major limitation to this approach is that only a restricted portion of the genome can be screened with 100% efficiency, namely the region encompassed by the balancer. In the case of RFB, using the *mxp*$^{Dch3}$ balancer, this may include as much as a single chromosome arm, or approximately 5% of the genome. However, because of the inbreeding scheme (involving father/daughter matings) incorporated into the extraction scheme, the remainder of the genome is also being screened, although at reduced efficiency. Balancers for other regions of the RFB genome are currently being developed in our laboratories. The second possible limitation is inherent in the mutagenesis approach, and does not specifically concern RFB. This limitation stems from the fact that mutagenesis is expected to generate primarily chromosome rearrangements or base

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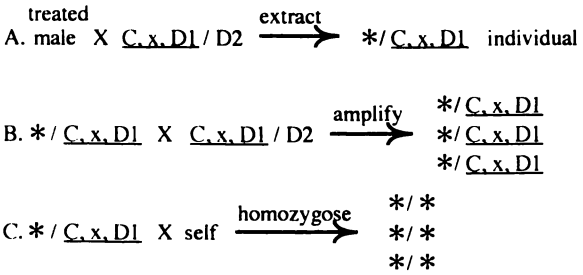

Figure 1. Generalized chromosome extraction procedure. (A) Extraction of a single mutagenized chromosome from a mutagenized (="treated") male by picking a single F1 adult. (B) Amplification of the mutagenized chromosome by single pair or single harem mating. (C) Homozygosing of the mutagenized chromosome by selecting against the D1 marker. This can be accomplished by self-crossing the appropriate F1 derived from cross (B) or by backcrossing appropriate daughters to the */C,x,D1 father to facilitate inbreeding. *, mutagenized chromosome; C, crossover suppressor; x, lethal mutation; D1, dominant visible mutation; D2, dominant visible mutation different from D1. In each cross the desired class can be phenotypically distinguished, and only this class is shown.

Table I. Capture of EMS-induced mutations by chromosome extraction using LG2 (Dch3) balancer

| type of mutation | No. screened | No. of genes found | frequency |
|---|---|---|---|
| lethal | 1607 | 7 | 0.004 |
| visible | 1607 | 7 | 0.004 |
| Total | 1607 | 14 | 0.009 |

1607 mutagenized LG2 chromosomes were screened for both visible and lethal mutations. Data refer only to distinct complementation groups.

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substitutions. Gene amplification-type resistance mechanisms may not arise directly by mutagenesis, although chromosomes carrying such variants can still be extracted from natural or laboratory populations.

## BT Susceptibility of *Tribolium castaneum*

As a prelude to attempting to screen for BT resistance mutations after chromosome extraction, we tested the susceptibility of a laboratory strain of RFB to ABG-6263, a beetle-active BT isolate designated BT subsp. *tenebrionis* (Abbott Laboratories, North Chicago, IL). ABG-6263 (lot #18-092-BR) was a spray-dried powder containing 860 Colorado potato beetle units/mg (determined by Abbott Laboratories). The inert ingredients consisted of fermentation solids, inert clays and various proprietary additives. A formulation blank containing only the inert ingredients was tested as a control. In order to ensure thorough mixing, we blended each formulations into flour as aqueous slurries, then lyophilized the suspension. The results (Table II) show that the threshold for toxicity to first instar larvae is between 0.1% and 1.0% BT in flour, calculated on the basis of total wt. of the BT prep. At toxic doses (1.0%) larvae are stunted and retarded in their development. Newly-hatched first instar larvae are the most susceptible stage, and susceptibility drops rapidly with larval age (unpublished observations).

When adults finally do develop they usually show a very specific and localized syndrome suggestive of juvenilization, namely retention of larval urogomphi. Normally these structures are present in larvae and pupae, but are entirely absent in adults. Adult survivors at this dose tend to show reduced fertility. A genetic mutant (termed *juvenile urogomphi*, or *ju*) that produces an identical syndrome, has been described and is fertile in both sexes (ref. *4* and unpublished observations). Retention of juvenile urogomphi in the adult is a well-known consequence of threshold doses of the juvenile hormone mimic, methoprene, in Tenebrionid beetles (*21*). Thus, it is possible that BT intoxication produces an indirect juvenilizing effect on beetle larvae. Such a possibility does not seem to explain the growth retardation seen in BT-intoxicated RFB larvae, since this effect of BT is evident throughout larval life.

Table II. Effect of BT on Tribolium exposed from the egg stage

| dose (%) | adults | pupae | larvae | F1 |
|---|---|---|---|---|
| 0 | 50 | 0 | 0 | >100 |
| 10C | 31 | 0 | 0 | 51 |
| .1BT | 28 | 0 | 0 | 0 |
| .3BT | 6* | 0 | 0 | 0 |
| 1BT | 2* | 0 | 0 | 0 |
| 3BT | 0 | 0 | 0 | 0 |
| 10BT | 0 | 0 | 0 | 0 |

Each datum (given as # of individuals) is total of 4 independent replicates. Two females were allowed to oviposit in each vial for three days. Vials were scored at day 60 for all develolpmental stages. C=control (formulation blank). BT=Bacillus thuringiensis formulation.
F1 refers to progeny of adults recovered at day 60.
*Retention of juvenile urogomphi in adults.

## Future Prospects

The widespread utility of chromosome extraction in RFB in the future will depend on the availability of new balancer chromosomes that cover larger regions of the genome, such as entire chromosomes. Pseudolinkage of nonhomologous balancer chromosomes by rearrangement might produce more complex balancers that could be used to simultaneously extract two or more chromosomes. Sets of recessive visible point mutations needed to detect and recover such rearrangements are available and are now being used in balancer screens.

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## Chapter 17

# Esterase Genes Conferring Insecticide Resistance in Aphids

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The amplified esterase genes responsible for insecticide resistance in the aphid *Myzus persicae* were remarkably conserved in standard laboratory clones and in samples collected from field populations throughout the UK during 1990. Only two restriction patterns were found, each associated with the presence of a different amplified esterase (E4 or FE4). The E4 and FE4 DNA sequences in the field aphids contained 5-methylcytosine (5 mC) at the same sites as in standard clones and although there were no examples of complete loss of methylation and esterase gene transcription, as previously found in revertant clones, there was evidence for partial loss of 5 mC and expression. An apparently identical esterase gene amplification and elevated enzyme (FE4) were found in a resistant Greek population of a closely related species, *Myzus nicotianae*.

The extensive use of chemicals to control insect pests worldwide has resulted in the development of resistance in many diverse insect species. At least 20 different aphid species have been shown by bioassay to be resistant to one or more insecticides, although evidence for the biochemical nature of the resistance is available for only a few (*1*). *Aphis gossypii* has become resistant to pirimicarb by developing a mutant, less sensitive form of the target protein, acetylcholinesterase, whereas *Myzus persicae* resists a range of insecticides by detoxification via increased carboxylesterase activity. Resistant *Phorodon humuli* also have increased esterase activity, although its role in resistance has not been established biochemically. The tobacco-feeding form of *M. persicae* which was recently classified as a new species, *Myzus nicotianae* (*2*), also resists organophosphorus insecticides by increased carboxylesterase activity (*3*). The molecular genetic basis of increased esterase activity in aphids is known only

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in the case of *M. persicae* (*4*) and this paper reviews the evidence for amplification and transcriptional control of esterase genes in this species and reports recent studies on the structure and methylation of esterase genomic sequences including homologous genes in *M. nicotianae*.

## Insecticide Resistance and Amplified Esterase Genes in *Myzus persicae*

A positive correlation between insecticide resistance and total carboxylesterase activity was first demonstrated in *M. persicae* over 20 years ago (*5*), and it was subsequently shown that only a single esterase is responsible and that the increased activity results from more esterase protein (*6*). Two forms of the esterase can confer resistance, E4 (a protein of *c* 65 kDa) present in resistant aphids with an A1,3 chromosome translocation or FE4 (*c* 66 kDa) found in aphids of apparently normal karyotype (*7*). An observed doubling in esterase content through a series of seven progressively more resistant *M. persicae* clones led Devonshire & Sawicki in 1979 (*8*) to propose gene amplification as the basis of the elevated enzyme synthesis. This was confirmed in 1989 when an E4 cDNA (pMp 24) was used to demonstrate increased esterase gene copy number in resistant aphids with either E4 or FE4 enzyme (*4*). Furthermore, the binding of the probe to *Eco*RI digested aphid DNA showed that amplified esterase sequences were present on an 8kb fragment in translocated aphids with E4 and a 4 kb fragment in aphids with FE4. This has now been demonstrated for many resistant clones from widely diverse origins and six examples are shown in Figure 1. In addition to the 4 kb and 8 kb fragments containing amplified FE4 and E4 sequences, there are other fainter bands in Figure 1, presumably resulting from binding of the E4 cDNA to non-amplified E4-related sequences. There is an apparent heterozygosity for the 10 and 15 kb fragments in translocated aphids (Figure 1, B samples) compared with homozygosity of the 10 kb fragment in aphids of normal karyotype (A samples). This marked conservation of restriction patterns between diverse aphid clones suggests that there is little polymorphism at the amplified E4 and FE4 loci.

The 4 kb and 8 kb *Eco*RI genomic fragments have been cloned and analyzed by restriction mapping using various cDNAs as probes (Field, L M and Devonshire, A L, Monograph of SCI meeting "Resistance '91" 1991, in press). This showed that E4 and FE4 genomic sequences are very similar and that the two genes probably differ only at the 3′ end, where there are both qualitative and quantitative variations. The findings are summarized in Figure 2B where the maps explain observed differences in *Msp*I digests in different aphid clones (see next section).

## Instability of Resistance in *M. persicae* Clones

In the absence of selection pressure, extremely resistant *M. persicae* clones with the A1,3 translocation can spontaneously lose resistance and elevated E4 between successive parthenogenic generations (*9*). Using E4 cDNA to probe

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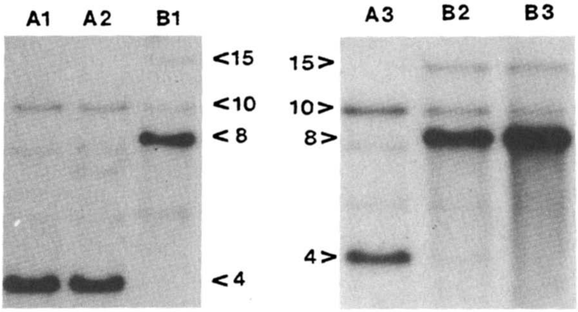

Figure 1. Binding of an E4 cDNA (pMp24)to Southern blots of *Eco*RI-digested DNA (10 μg) from 6 different insecticide-resistant *M. persicae* clones with either A. Normal karyotype and FE4 enzyme or B. Translocated karyotype and E4 enzyme. Clones A1 and A2 were established with aphids collected from peach trees in Italy and France respectively, whilst A3 originated on field potatoes in the UK. The three B clones originated in the UK, two in glasshouses (B1 and B3) and the third (B2) in a sugar beet field. Arrows indicate sizes of the major fragments in kilobases.

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nucleic acids from such "revertant" clones has shown that they lose elevated E4 mRNA but retain the amplified E4 genes with no major DNA rearrangement (*10*). Thus the basis of reversion is a change in expression of the amplified E4 genes. So far the only difference observed between the expressed and silent E4 sequences is a change in the presence of 5-methylcytosine (5 mC) (*10*), a fifth base that is known to affect gene transcription in vertebrates (*11*). The presence of 5 mC in aphid esterase DNA sequences can be demonstrated by comparing the fragments produced when aphid DNA is cut with *Msp*I or *Hpa*II and probed with E4 cDNA. Both *Msp*I and *Hpa*II cut CCGG sites but only *Msp*I will cut if the internal cytosine is methylated (i.e. is a 5 mC). Thus a difference in banding pattern between the 2 enzyme digests, as shown in Figure 2A for $R_1$ and $R_3$ aphids indicates the presence of 5 mC, in both FE4 and E4 sequences whereas the identical *Msp*I and *Hpa*II digests of DNA from the revertant aphid clone (Rev) show that in the silent E4 DNA sequences the CCGG sites are not methylated. This correlation holds for a large number of aphid clones. It is very surprising in the light of many studies with vertebrate genes where DNA methylation has been shown to correlate with gene inactivation and demethylation is a necessary prerequisite for transcription to occur (*11*).

The banding patterns in Figure 2A can be explained by the restriction maps in Figure 2B. Thus the *Msp*I digest of $R_1$ (FE4 genes) reveals amplified esterase sequences on 2.8 and 1.8 kb fragments, whereas $R_3$ and Rev both with amplified E4 genes have 2.8 and 2.2 kb bands. When the DNA is methylated in $R_1$ and $R_3$, the small fragments are absent in the *Hpa*II digests indicating that at least two of the *Msp*I sites in both E4 and FE4 sequences must contain 5 mC; in the revertant the 3 *Msp*I sites in the E4 sequences must lack methylation. Since the 2.8 kb fragment is common to both genes the E4 cDNA probe will have equal homology to this region and the difference in intensity between $R_1$ and $R_3$ reflects differences in gene copy number. However, the homology of the E4 cDNA to fragments downstream of the *Sma*I site, where the 2 genes differ, should be greater for the E4 gene (2.2 kb fragment) than for the FE4 (1.8 kb fragment), and this can be seen clearly in Figure 2A. To avoid this complication in studies of esterase genes in aphids from field and glasshouse populations, we have subcloned the genomic fragments shown in Figure 2B to use as probes.

## Esterase Genes in UK Field Populations of *M. persicae*

When revertant aphid clones are subjected to insecticide treatment, resistance can be re-selected very quickly (*12*), and therefore it is important to know to what extent revertants occur in the field. We have used the characteristic *Msp*I and *Hpa*II banding patterns to identify the type of amplified esterase genes present, and their methylation, in samples of *M. persicae* taken from populations in the UK during 1990, and have made preliminary attempts to relate this to the expression of the genes as judged by immunoassay (*13*).

Individual aphids were placed on potato leaves in small plastic boxes,

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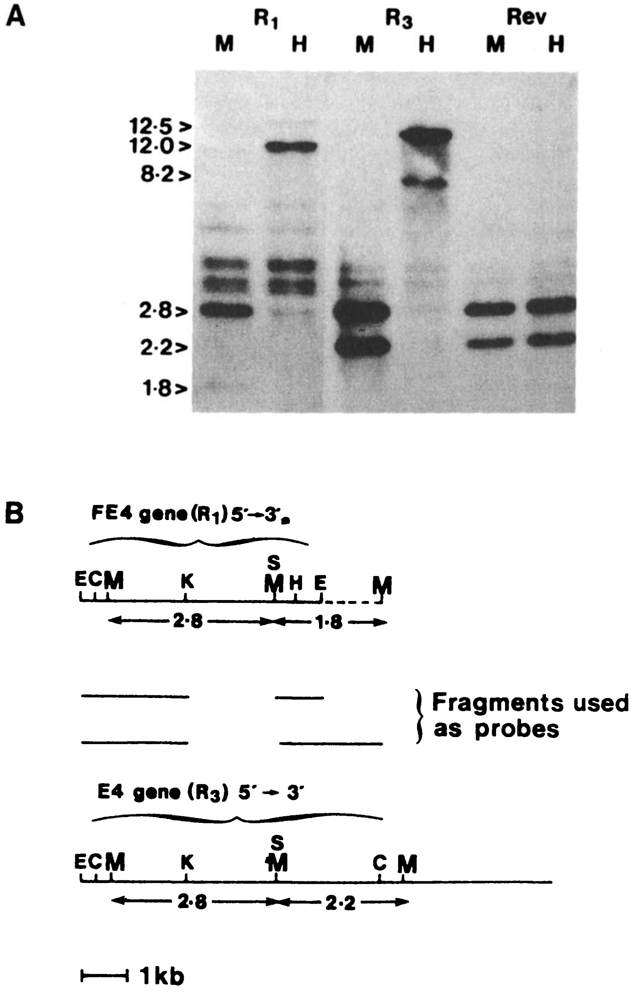
A
R1
R3
Rev
M
H
M
H
M
H
12·5 >
12·0 >
8·2 >
2·8 >
2·2 >
1·8 >
B
FE4 gene (R1) 5'→3'
E C M
K
S
M H E
M
2·8
1·8
Fragments used as probes
E4 gene (R3) 5'→3'
E C M
K
S
M
C M
2·8
2·2
1kb

Figure 2. A. Binding of an E4 cDNA (pMp24) to Southern blots of *Msp*I (M)- or *Hpa*II (H)-restricted DNA from resistant *M. persicae* clones with expressed FE4 ($R_1$) or E4 ($R_3$) genes and a revertant clone (Rev) with unexpressed E4 genes. Arrows indicate sizes of the major fragments in kilobases. B. Restriction maps of the 4 kb and 8 kb *Eco*RI fragments in $R_1$ and $R_3$ aphids containing amplified FE4 and E4 sequences respectively.

E = *Eco*RI C = *Cla*I M = *Msp*I K = *Kpn*I S = *Sma*I H = *Hind*III

Restriction at the M sites in 2B gives the *Msp*I fragments seen in 2A (see text for details).

DNA was extracted from all of their first generation clonal offspring (*c* 20 aphids) and pairs of *Msp*I and *Hpa*II digests were probed with a mixture of the esterase genomic sequences described above. The aphids used to establish these single generation "miniclones" were from diverse populations. In the examples shown in Figure 3, samples 5 and 6 were collected from a potato field in Scotland and the remainder originated in England; 1-3 and 9 from field crops of potatoes, cauliflowers, sugar beet and fodder beet respectively, sample 4 from sugar beet in a glasshouse and samples 7 and 8 from insect suction traps.

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In all, 84 samples were analysed; most (75), had amplified esterase genes, 25 with E4, as judged by the presence of 2.8 and 2.2 kb *Msp*I fragments, (e.g. samples 1-4, Figure 3) and 50 with FE4, having 2.8 kb and 1.8 kb *Msp*I fragments (e.g. samples 5-8, Figure 3). There were no cases of aphids with both 2.2 and 1.8 kb bands, suggesting that amplified E4 and FE4 genes do not co-exist in an individual insect. In the majority (66) of samples with amplified esterase sequences the small fragments were absent in the *Hpa*II digests (i.e. like samples 1, 2 and 5, 6 Figure 3) indicating methylation of the CCGG sites discussed above. In all such cases the immunoassay showed correspondingly high esterase activity. However, some samples retained the small fragments in the *Hpa*II tracks even though most of their amplified esterase sequences appeared on larger fragments (e.g. samples 3, 7 and 8 Figure 3); this suggests that both E4 and FE4 DNA sequences can be "partially" methylated at the 3 CCGG sites, perhaps by differential methylation between cells, tissues and/or embryos, or perhaps between individuals within the miniclones. This partial methylation correlated with enzyme concentrations less than expected from the amount of probe binding although this could not be quantified accurately; thus both E4 and FE4 genes seem to be partially silenced in these aphids. This is particularly interesting for aphids with amplified FE4 genes since reversion has not been observed in aphid clones with elevated FE4.

The miniclone established from a glasshouse sample (sample 4 Figure 3) was clearly a revertant with E4 genes as judged by the 2.8 and 2.2 kb fragments in the *Hpa*II digest and by the presence of an esterase concentration typical of susceptible aphids even though the amount of probe binding suggests a high degree of E4 gene amplification. This is consistent with our previous finding that glasshouse aphids with extremely high levels of resistance and amplified E4 genes frequently revert, although changes in their DNA methylation were not studied (*14*). None of the samples from field populations showed this complete loss of 5 mC, but this may be more likely to occur as extremely resistant aphid variants become more common in the field.

Nine of the samples did not have amplified esterase DNA or elevated enzyme (e.g. sample 9, Figure 3) and were deemed to be susceptible types. In all 9 clones the 2.8 kb band was present in both *Msp*I and *Hpa*II digests but neither the 2.2 nor the 1.8 was detected, showing that esterase sequences in susceptible field aphids are unmethylated (as they are in susceptible clones, *10*) and suggesting that the original unamplified esterase gene and/or flanking DNA in susceptible aphids may differ from the amplified E4 and FE4 DNA sequences.

All of the field aphids with amplified esterase sequences had one of the two restriction patterns found in resistant clones, again supporting the view that there is little polymorphism at these amplified loci.

## Esterases and their DNA Sequences in *M. nicotianae*

Blackman (*2*), using multivariate morphometric techniques, showed that the tobacco-adapted form of *M. persicae* should be considered as a separate species, *M. nicotianae*. He also showed that *M. nicotianae* populations in the USA frequently had a heterozygous A1,3 chromosome translocation, apparently the same as that present in *M. persicae*. He suggested that this had arisen independently since the separation of the two species, occurring either just after or at the same time as the acquisition of insecticide resistance by *M. nicotianae*. This suggested that *M. nicotianae* might have the same insecticide resistance mechanism as *M. persicae*, a hypothesis supported by recent publications on the esterases of both species in the USA (*3*).

We have examined insecticide resistant fundatrigeniae from peaches and summer populations of aphids from tobacco in Greece, identified as *M. nicotianae* of normal karyotype (Blackman, Pers Comm). Gel electrophoresis patterns of individual *M. nicotianae* (samples 2-8, Figure 4) were indistinguishable from that of a non-translocated very resistant *M. persicae* clone (800 F,sample 1, Figure 4) from Ferrara (*7*), showing the same range of non-amplified esterases (E1/2, E5, E6 and E7) and similar levels of the elevated esterase, FE4. DNA probing with E4 and FE4 genomic sequences (Figure 4B) showed that the *M. nicotianae* contained homologous amplified esterase sequences on fragments consistent with the presence of the same FE4 genes as found in *M. persicae*, i.e. 2.8 and 1.8 kb bands in *Msp*I digests. Furthermore, the *Hpa*II digests gave a dominant 12 kb band showing that the esterase sequences in *M. nicotianae* are methylated in the same way as in resistant *M. persicae*. Thus the molecular genetic basis of insecticide resistance appears to be the same in both species.

It is possible that the insecticide resistant *M. nicotianae* in this study evolved from resistant *M. persicae* and therefore the amplified esterase genes were present before the divergence of the two species. Alternatively, insecticide resistance in the Greek population may have evolved after they separated from *M. persicae*, as suggested by Blackman (*2*) for the translocated USA populations, in which case two independent amplification events must have occurred. Since the amplified sequences in *M. persicae* and *M. nicotianae* appear to be identical it would suggest that aphids are somehow "predisposed" for a particular amplification event. If so, then the same amplification event could also have occurred more than once in *M. persicae* which would explain the observed, conserved sequences in clones of widely different origin.

This would be in contrast to the situation for insecticide resistant culicine mosquitoes where there is evidence that a single esterase B2 gene amplification event occurred and that this was subsequently spread worldwide

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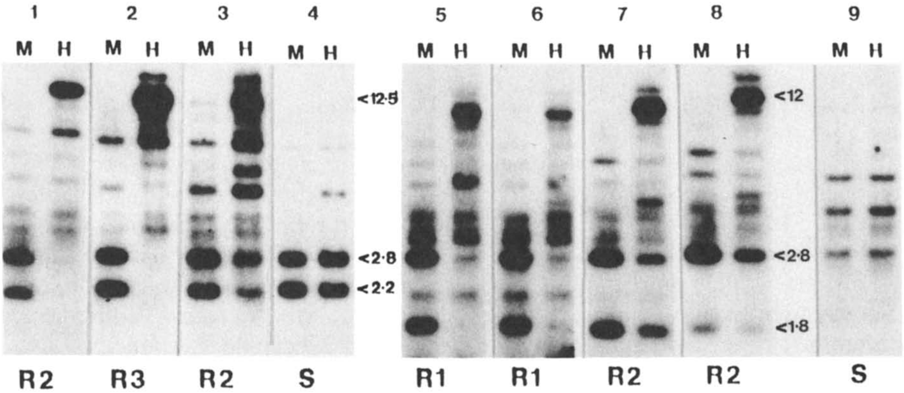

Figure 3. Binding of a mixture of esterase genomic fragments (see Figure 2) to Southern blots of DNA (2 μg) extracted from 9 *M. persicae* "Miniclones" and digested with either *Msp*I (M) or *Hpa*II (H). Arrows indicate sizes of major fragments in kilobases. Lower line gives the level of esterase in the clone as judged by immunoassay.

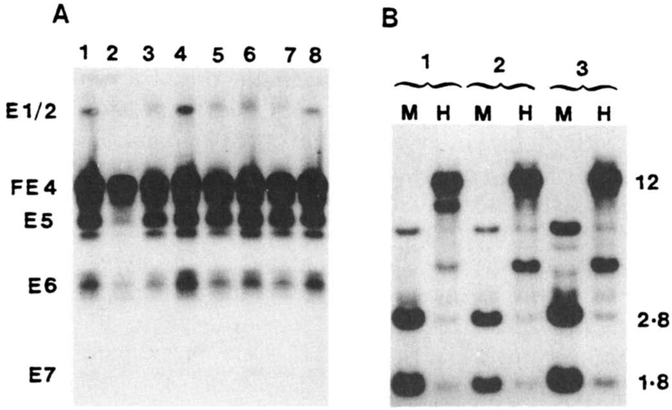

Figure 4. A. Polyacrylamide gel electrophoresis of individual aphid homogenates stained for esterase activity using 1-naphthyl acetate (*6*). Sample 1 is a nontranslocated resistant *M. persicae* standard (Ferrara) and samples 2–8 are *M. nicotianae*. B. Binding of mixed esterase genomic fragments (see Figure 2) to Southern blots of DNA (2μg) extracted from 3 resistant *M. nicotianae* clones (of normal karyotype) and digested with *Msp*I (M) or *Hpa*II (H). Indicated are the sizes of major fragments in kilobases.

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by migration (*15*). The example of gene amplification in mosquitoes is the only other confirmed case of insecticide resistance arising from esterase gene amplification, although there is circumstantial evidence in several other species. As in *M. persicae*, resistant mosquitoes can show amplification of various esterase alleles but there is no evidence for transcriptional control of the genes nor for methylation of the DNA sequences. Similarities and differences between aphid and mosquito resistance are discussed fully in a recent review of gene amplification and insecticide resistance (*16*).

## Acknowledgements

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## Chapter 18

# Insecticide Resistance Mechanisms in the German Cockroach, *Blattella germanica* (L.)

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Insecticide resistance in the German cockroach has been identified for all major classes of insecticides currently in use. Resistance mechanisms that have been identified include increased metabolic detoxification, decreased penetration and target site insensitivity. Resistance to pyrethroid insecticides involves a *kdr*-type nerve insensitivity and possibly increased oxidative detoxication. Organophosphate and carbamate resistance mechanisms include increased hydrolytic and oxidative metabolism but do not involve an altered or insensitive acetylcholinesterase. Metabolic detoxification, when identified, involves a complex of enzyme systems which act in concert to produce significant resistance levels. Resistance mechanisms identified for chlorpyrifos and propoxur indicate that different forms of both oxidative and hydrolytic enzymes are responsible for the resistance. Implications to management and cross resistance are discussed.

The German cockroach, *Blattella germanica*, is perhaps the most important urban pest species across the U.S. In addition to the nuisance associated with large populations in household settings, German cockroaches are considered to be serious pests of economic and medical importance because: 1) they carry and transmit a large number of disease organisms, notably *Salmonella*, but also including several viruses, pathogenic bacteria and pathogenic helminths, 2) they are responsible for severe allergic reactions due to their feces and debris, 3) they are omnivorous, and very little food is needed to sustain large populations, and 4) cockroaches are frequently associated with food handling establishments, and food fouled and tainted by the characteristic odor of cockroaches is unfit for human consumption (*1*). Insecticides have provided the only effective control of German cockroaches for the last 40 years, although resistance in *B. germanica* has become a substantial problem that contributes to control failures in many areas of the country.

Resistance in German cockroaches has been demonstrated to a wide range of insecticides including organochlorine, organophosphate, carbamate and most recently, pyrethroid insecticides. Field monitoring has been successful in documenting the widespread nature of resistance in this species (eg. *2-5*), but very little information is available on the responsible mechanisms. Resistance in the

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German cockroach was first identified for chlordane and DDT (*6,7*) in the early 1950s . Resistance to chlordane rapidly became so widespread that this compound was no longer considered an effective control agent, and the pest control industry turned to other insecticides such as the organophosphates. The importance of insecticide resistance in cockroaches became obscured in the 1960s and early '70s due to the excellent control provided by OP and carbamate insecticides then in use (*8*), although reports of low to moderate resistance to several OP insecticides and high-level resistance to malathion were beginning to appear (*7*). The incidence of resistance was not widespread, however, and when problems did occur, they were easily circumvented by rotation to other compounds. Presently, OP and carbamate insecticides provide adequate control in some areas, although moderate levels of resistance commonly occurs to diazinon, fenthion, chlorpyrifos, malathion, and propoxur, and high levels of resistance to malathion, propoxur, and bendiocarb have been sporadically reported throughout the U.S. (*8,9*). Resistance to pyrethroid insecticides has also been detected in a number of field populations (*2,10*) despite the more recent introduction of these compounds for German cockroach control.

The mechanisms of insecticide resistance in *B. germanica* have received little attention relative to other insect pests. Although resistance is considered to be a serious problem in this species, satisfactory control has usually been achieved by choice of an appropriate compound (*2,8*). However, with the increasing severity of the resistance problem and recognition for the need to identify resistance mechanisms in order to implement resistance management programs, a more thorough understanding of the nature of resistance in this species is slowly emerging. In addition to the benefits associated with mechanism identification and resistance management, German cockroaches have several characteristics that make them especially useful in the study of resistance mechanisms: 1) their relatively large size allows isolation of discrete tissues, 2) large numbers can be reared with minor investment in labor and materials and 3) their phylogenetic position makes them one of the oldest insect taxa for which resistance has been detected so that information about evolutionary origins of resistance within the class Insecta can be obtained. Perhaps the biggest hindrance to their use in the study of insecticide resistance is a relatively long generation time [ca. 3 months from egg to adult (*11*)] such that crossing and back-crossing experiments require long periods of time.

The following discussion presents the current state of knowledge regarding physiological mechanisms of resistance to compounds representing the major classes of insecticides that have been used for German cockroach control; organochlorine, organophosphate, carbamate and pyrethroid insecticides. Known physiological mechanisms of insecticide resistance in insects include target site modification or insensitivity, decreased rates of penetration, and increased metabolic detoxification (*12*), and the following discussion has been subdivided according to these generally accepted resistance mechanisms. All of these insecticide resistance mechanisms have been implicated to some extent in German cockroaches.

## Target Site Insensitivity.

Because most of the commonly used insecticides for German cockroach control represent compounds that are neurotoxic agents, the following discussion focuses on components of the central nervous system which have been identified as target sites for these compounds and the modifications to this system that have occurred as a result of insecticide selective pressures.

**Acetylcholinesterase.** Acetylcholinesterase (AChE) is an important regulatory enzyme responsible for controlling the transmission of nerve impulses across cholinergic synapses where it acts to hydrolyze the excitatory neurotransmitter

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acetylcholine. AChE in insects has important toxicological significance because it is readily inhibited by organophosphate (OP) and carbamate insecticides commonly used in their control. Modification of AChE has been implicated in OP and carbamate resistance mechanisms for a number of important medical, veterinary, and agricultural insect and acarine pest species (*12,13*). In German cockroaches, the role of AChE in resistance to OPs and carbamates is still uncertain, although the limited information available suggests that it is probably of minor importance.

Mansingh (*14*) conducted a set of experiments with malathion resistant *B.germanica* to determine the *in vivo* inhibition of AChE by topically-applied malathion. Substantial differences in the rate of AChE inhibition were evident between resistant and susceptible strains. It was concluded that there was a mechanism that protected AChE or reactivated the enzyme in the resistant strain. In a similar study with multi-resistant cockroaches, *in vivo* inhibition again showed elevated AChE activity relative to a susceptible strain (*15*). However, *in vitro* determinations of AChE inhibition for resistant and susceptible cockroaches indicated similar values for the molar concentration of insecticide necessary to inhibit 50% of the activity. This strain also exhibited increased rates of both malathion and carbaryl metabolism, and it was concluded that resistance was due to enhanced levels of metabolic detoxification rather than AChE insensitivity.

In a study in which 4 resistant strains displaying varying levels of both carbamate and OP resistance were compared with a susceptible laboratory strain, it was determined that inhibition rates (based on the bimolecular rate constants, $k_i$) for three AChE inhibitors (malaoxon, chlorpyrifos oxon, and propoxur) did not differ among the resistant and susceptible strains (Table I) (*16*). Although slight variation in the bimolecular rate constants were observed, this variation was minor in comparison to resistance levels and not on the order of magnitude reported in other insect species where AChE insensitivity has been implicated (*13*). That insensitivity of AChE is not an important factor conferring resistance in several cockroach strains collected from widely different locations and probably resulting from different selection pressures suggests that AChE insensitivity is very rare in this species. Alterations within the central nervous system have been associated with both pyrethroid and cyclodiene resistance (see below) so that a precedence exists in this species for modified CNS target sites that result from insecticide selection pressures. However, it does not appear that German cockroaches commonly possess the genetic plasticity that would be necessary for modification of AChE to occur.

**Cyclodiene Resistance.** A major mechanism by which insects may become resistant to cyclodiene insecticides is nerve insensitivity. This mechanism provides cross resistance to all cyclodienes (and related chlorinated hydrocarbon insecticides), and has been linked to a single major gene. Based on cross-resistance to the $\gamma$-aminobutyric acid antagonist, picrotoxinin, and a lower [$^3$H] $\alpha$-dihydropicrotoxinin binding capacity in the resistant strain, it appears that the mechanism of resistance to cyclodiene insecticides in German cockroaches is due to an altered picrotoxinin receptor (*17*). It is encouraging to note that cyclodiene resistance in this species does not appear to cause cross resistance to abamectin (J.G.S. unpublished observation) even though these compounds may act at the same general target site (*18-21*).

***kdr* Nerve Insensitivity.** One of the most important mechanisms by which insects become resistant to pyrethroids is known as *kdr* (knock-down resistance due to insensitivity of the nervous system). First described in house flies (*22*), this type of resistance can confer cross resistance to essentially all pyrethroids (and DDT), thus greatly reducing the effectiveness of an entire class of insecticides. In 1981, Scott and Matusmura (*23*) demonstrated that a *kdr*-type mechanism was responsible for resistance to DDT and cross resistance to pyrethroids in a DDT selected strain of

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German cockroach (VPIDLS). However, resistance was not stable in this strain and it constantly reverted to susceptibility in the absence of selection pressure (J.G.S. unpublished observation). Selection of the VPIDLS strain with permethrin resulted in a strain (Ectiban-R) which no longer reverts to susceptibility (*24,25*). The lack of reduction in permethrin resistance in this strain with metabolism inhibitors (*24*), and similar rates of both permethrin metabolism and cuticular penetration of deltamethrin in VPIDLS and Ectiban-R (*25*) suggests that resistance in Ectiban-R is solely due to the *kdr*-type mechanism. The identification of *kdr*-type resistance in German cockroaches from the United States (*23*) and Japan (*27*) suggests that *kdr*-type resistance may be a problem for control of this pest throughout the world.

**Table I. Bimolecular rate constants, $k_i$, for inhibition of acetylcholinesterase and respective resistance levels for five strains of German cockroaches**

| *Strain* [a] | *Inhibitor* | $10^{-3} \times k_i$ $(M^{-1}min^{-1})$ | *S/R* [b] | *Resistance Ratio* [c] |
|---|---|---|---|---|
| CSMA | Chlorpyrifos oxon | 239 ± 16 | -- | -- |
| Dursban-R | " | 254 ± 17 | 0.94 | 20.2 |
| CHL | " | 188 ± 7.2 | 1.27 | 8.3 |
| Kenly | " | 196 ± 14 | 1.22 | 4.6 |
| Rutgers | " | 213 ± 11 | 1.12 | 3.4 |
| CSMA | Malaoxon | 126 ± 6.4 | -- | -- |
| Dursban-R | " | 113 ± 5.6 | 1.11 | >60 |
| CHL | " | 114 ± 6.3 | 1.10 | >50 |
| Kenly | " | 128 ± 4.2 | 0.98 | 5.3 |
| Rutgers | " | 121 ± 5.9 | 1.04 | 24.2 |
| CSMA | Propoxur | 115 ± 5.6 | -- | -- |
| Dursban-R | " | 97.5 ± 6.0 | 1.18 | 5.0 |
| CHL | " | 110 ± 4.2 | 1.04 | 10.5 |
| Kenly | " | 109 ± 4.7 | 0.95 | 157 |
| Rutgers | " | 106 ± 6.0 | 0.92 | 5.3 |

[a] Acetylcholinesterase preparations from adult male German cockroach heads (20 heads/ml buffer).

[b] Ratio of bimolecular rate constants for susceptible (CSMA)/resistant strains.

[c] Resistant ratio = $LD_{50}$ of resistant / $LD_{50}$ of susceptible strain. Resistance ratios for Dursban-R strain from Siegfried *et al.* (26) and for Kenly and Rutgers from Scott *et al.* (*24*). Resistance ratios for CHL determined as previously described (*24*). For chlorpyrifos oxon and malaoxon, the resistance ratios determined for the corresponding phosphorothioates.

The *kdr*-type resistance in Ectiban-R is autosomal, incompletely recessive and probably monofactorial (*25*). Ectiban-R is cross-resistant to bioallethrin (48-fold), deltamethrin (17-fold), fenvalerate (59-fold), aconitine (16-fold), batrachotoxin (8.7-fold) and verapamil (5.4-fold), but not to other sodium channel drugs or other neurotoxins acting elsewhere in the nervous system (*25*). Using $^3H$-saxitoxin as a probe of the sodium channel, Dong and Scott (*25*) found no difference in binding affinity or number of binding sites per head (or per mg protein) between resistant and susceptible strains. These results suggest that the sodium channels of *kdr*-type resistant German cockroaches are qualitatively different than those of the susceptible strain.

Genetic analysis suggests that the genes for *kdr*-type resistance and black body color (on linkage group VI) are not linked (*25*). Cochran (*28*) showed that pyrethrins resistance in the R-Pyr strain of *Blattella germanica* was associated with the gene for pallid eye on linkage group VI. As pallid eye and black body are tightly linked on linkage group VI (*29*) it appears that *kdr*-type gene in Ectiban-R is different from the gene responsible for pyrethrins resistance in the R-Pyr strain (*25*). Interestingly, the number of both wild type and hybrid body color cockroaches surviving at a discriminating dose were less than expected (*25*). This suggests that some fitness disadvantage occurs with the *kdr*-type gene, which is consistent with the observation that DDT resistance in VPIDLS (parental strain of Ectiban-R) reverted quickly when selection pressure was stopped.

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## Penetration Barriers

Penetration barriers only have moderate effects on resistance levels in insects in the absence of other resistance factors such as metabolism and target site insensitivity (*12*). In German cockroaches, penetration barriers that prevent or reduce the rate of insecticide movement across the integument have been implicated in resistance for a number of strains. However, in most of these instances, other mechanisms have been shown to contribute to resistance levels so that it is not possible to determine the overall effect of penetration in the absence of other factors. Resistance to carbaryl is partially associated with retarded penetration based on the increased levels of radioactivity in surface rinses of resistant roaches treated topically with $^{14}$C-carbaryl relative to a susceptible strain (*30*). This resistant strain also exhibited increased carbaryl hydrolysis and conjugation of carbaryl metabolites, and the role of penetration in overall resistance levels was considered minor.

A penetration barrier has also been associated with propoxur resistance in a multi-resistant strain of cockroaches (*31*) based again on insecticide loss from surface rinses over time of topically treated cockroaches (Figure 1). Resistance to topically applied propoxur was approximately 11-fold, but decreased to less than 6-fold when the insecticide was injected. The decline in resistance associated with insecticide injection vs topical application confirms the influence of penetration barriers on resistance. However, since significant resistance was detected in the absence of integumental barriers to penetration, it was concluded that other resistance factors also contribute to the resistance. Comparisons of topically applied vs injected diazinon and DDT have also implicated penetration barriers in a strain of cockroaches selected with diazinon (*32*). Resistance to topically applied diazinon was 4-fold higher than injected and 20-fold higher for DDT. Some specificity for this form of penetration barrier was suggested by the lack of differences noted for susceptibility to topically-applied and injected propoxur and pyrethrins. It should be noted that penetration barriers are not universally present in resistant *B. germanica*. Similar penetration studies with chlorpyrifos resistant and susceptible strains resulted in identical penetration curves (*26*).

Of interest with regard to the presence of a penetration barrier in German cockroaches, is the possibility that it may confer cross resistances. Recent reports of resistance to topically applied abamectin in German cockroaches may involve a penetration barrier since both strains for which abamectin resistance has been reported (*33*) are closely related to a strain for which a penetration barrier has been identified

(*31*). Resistance to abamectin as a result of reduced penetration may explain the lack of resistance noted for injested abamectin in several multiresistant strains (*34*).

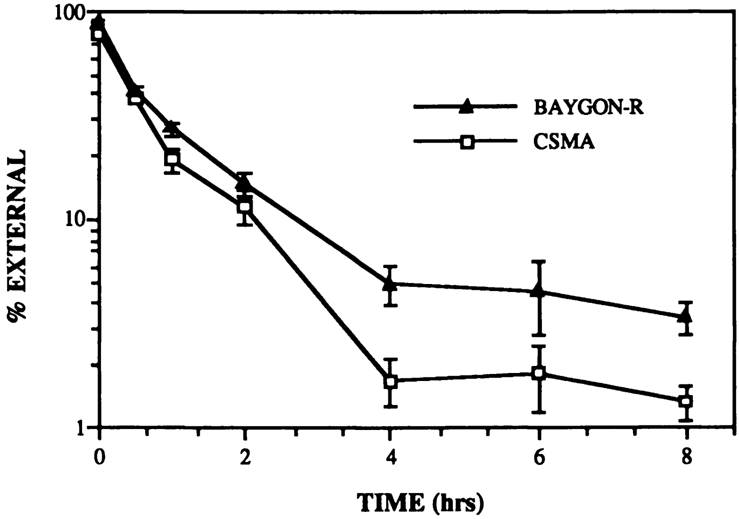

Figure 1. Penetration of $^{14}$C-propoxur in resistant and susceptible German cockroaches

## Metabolic Detoxification

The role of metabolism in resistance of German cockroaches to synthetic insecticides has been established for only a few compounds, although the information available suggests that metabolic resistance is fairly widespread and is involved in resistance to pyrethroid, OP and carbamate insecticides. The two enzymatic systems most commonly implicated include the cytochrome P450-dependent monooxygenases and hydrolytic enzymes, although involvement of specific enzyme systems is often based on indirect evidence. Gutathione transferase and other conjugative enzymes have not been shown to contribute to resistance in German cockroaches thus far.

**Synergism Studies.** Much of the evidence implicating metabolic detoxification in the resistance mechanisms of *B. germanica* is based mainly on synergism studies. These studies rely on compounds which inhibit specific detoxifying enzymes thereby affecting resistance in treated insects if the mechanism involves the respective enzyme (*35*). Cochran (*36*) used two cytochrome-P450 monooxygenase inhibitors, piperonyl butoxide (PBO) and MGK 264, to determine their effect on resistance in several bendiocarb and pyrethrins resistant strains of German cockroach. Both synergists effectively negated resistance to each compound, and therefore, resistance was attributed to an enhanced microsomal monooxygenase system. Synergism studies with PBO and the hydrolytic enzyme inhibitor DEF (*S,S,S*-tributylphosphortrithioate) were used to obtain preliminary information on resistance mechanisms in three German cockroach strains, two of which are multiresistant to OP, carbamate and pyrethroid insecticides and a third with resistance specific to certain pyrethroids (*24*). Resistance to cypermethrin in the pyrethroid resistant strain was unaffected by PBO and DEF (Figure 2), suggesting that resistance is not the result of increased metabolic detoxification but rather a *kdr*-type mechanism (*23*).

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High levels of resistance to propoxur and bendiocarb in the multiresistant strains were partially suppressed by both synergists (Figure 2) suggesting that the combined effects of oxidative and hydrolytic metabolism are, at least in part, responsible for this resistance. Similarly, involvement of oxidative and hydrolytic enzymes has been implicated in chlorpyrifos and propoxur resistance (*26,31*). High malathion resistance (>300-fold) was completely suppressible by PBO but was unaffected by DEF suggesting that oxidative metabolism was solely responsible for the resistance. Resistance to pyrethrins in one of the multiresistant strains was largely unaffected by either compound (Figure 2).

This result contrasts with a that of a time-variable residual contact bioassay in which resistance to pyrethrins was reduced from >80- to 1.3-fold with PBO (*36*).

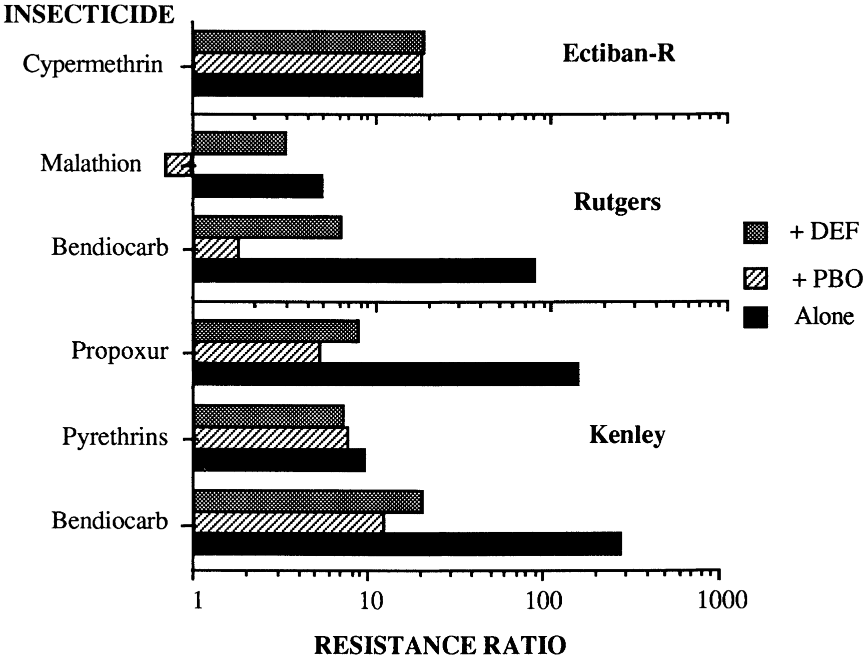

Figure 2. Effect of pre-treatment with PBO or DEF on insecticide resistance in three strains of German cockroach.

This discrepancy may be the result of differences in bioassay techniques since one study exposed insects by topical application and the other used residual exposure. With the time-mortality method, immobilization (end-point in this bioassay) of the susceptible strain is very rapid because a relatively high concentration of insecticide is used. The rate of cuticular penetration would overwhelm that of metabolism and determine interaction with the target site (*24,37-39*). Thus, addition of a metabolic inhibitor would have little or no effect on the $LT_{50}$ of the susceptible strain. However, in the resistant strain, longer times are needed for immobilization, and metabolism is again important in the poisoning process. Therefore, synergism can be observed only in the resistant strain, giving the illusion that the synergist reduced resistance levels. Alternatively, the two different methods of application may affect different physiological systems, resistant mechanisms, or both. The involvement of metabolic detoxification in pyrethroid resistance has been further investigated by the use of synergists (PBO and DEF) in a highly pyrethroid resistant strain (> 300-fold to fluvalinate) (*10*). In this study, both synergists were effective in reducing the level of resistance suggesting that both oxidative and hydrolytic metabolism contribute to resistance. However, neither compound completely negated resistance and the authors concluded that target site insensitivity was also involved in resistance for this strain.

It is apparent from the preceding discussion that generalizations regarding mechanisms of pyrethroid insecticides are inappropriate, and the possibility exists for multiple resistance mechanisms. Therefore, strains where pyrethroid resistance is documented should be examined individually to determine the exact nature of the resistance mechanism.

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***In vivo* Metabolism.** While the synergism studies discussed above provide indirect evidence for involvement of metabolic detoxification in a resistance mechanism, the most conclusive evidence has been achieved by directly measuring the rate of insecticide detoxification using *in vivo* and *in vitro* techniques. Unfortunately, only a limited number of these studies have been conducted with German cockroaches and broad generalizations are difficult to formulate. Two such studies have been conducted with malathion and carbaryl. Ku and Bishop (*30*) found that both resistant and susceptible cockroaches hydrolyzed $^{14}$C-carbaryl *in vivo* based on the production of 1-naphthol and 1-naphthol conjugates. However, the resistant strain exhibited slower carbaryl penetration, lower levels of carbaryl in tissue extracts, and higher rates of excretion. Slightly larger amounts of internal and excreted 1-naphthol were found in the susceptible cockroaches but more conjugated 1-naphthol was recovered from resistant cockroaches. The authors hypothesized that more rapid hydrolysis of carbaryl to 1-naphthol and subsequent conjugation play an important role in the carbaryl resistance and slower penetration and more rapid excretion were minor contributing factors to overall resistance levels.

Bull et al. (*15*) compared absorption, metabolism, internal accumulation, and excretion of topically applied $^{14}$C-carbaryl and $^{14}$C-malathion in multiresistant and susceptible cockroaches and found no significant differences between the two strains. Additionally, it was shown that malathion and carbaryl were rapidly metabolized by both strains, although no qualitative or quantitative differences in the distribution of parent compounds or their metabolic products in the internal extracts or excreta were apparent. However, metabolism studies using injected carbaryl and malathion demonstrated that metabolic degradation of both compounds was substantially enhanced in the resistant strain. The authors suggested that lack of differences observed in topical tests were related to the sublethal doses of insecticide used to treat both strains. Penetration rates were slowed to the extent that the concentrations of malathion or carbaryl entering the insect's body were insufficient to challenge the detoxifying enzyme systems to show differences between strains in metabolic capacity.

Although both of the previous studies are fairly conclusive with regard to implicating metabolic detoxification in resistance, actual rates of specific enzymatic reactions were not measured. Complete resistance mechanisms involving metabolic detoxification have been identified for only two strains of cockroaches (*26,31*). The Dursban-R strain is multiresistant to OP, carbamate, and pyrethroid insecticides although highest levels of resistance have been reported for organophosphates such as chlorpyrifos, parathion and malathion. The Baygon-R strain is also multiresistant with highest levels of resistance noted for carbamates such as bendiocarb and propoxur. Both strains exhibit PBO and DEF suppressible resistance suggesting involvement of oxidative and hydrolytic metabolism in resistance. Additionally, *in vivo* experiments using $^{14}$C-chlorpyrifos and $^{14}$C-propoxur for the Dursban-R and

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Baygon-R strains, respectively, implicated increased insecticide metabolism in both resistant strains. In Dursban-R, increased metabolic detoxification relative to susceptible strain was indicated by decreased levels of chlorpyrifos recovery, increased metabolite formation, and perhaps most importantly, reduced levels of chlorpyrifos oxon, the active form of chlorpyrifos. Parallel studies with Baygon-R indicated that propoxur was metabolized at higher rates based on reduced levels of propoxur recovery, although higher levels of metabolites were recovered from the susceptible strain. This apparent discrepancy may have been the result of subsequent metabolism of primary metabolites, since total recovery of radioactivity was also reduced in the resistant strain. Alternatively, hydrolytic metabolism of propoxur could result in metabolites that enter into pathways eventually resulting in expired carbon dioxide (*39*) thus explaining the reduced levels of total recovery.

***In vitro* Metabolism.** Although *in vivo* metabolism studies are fairly conclusive in associating metabolic detoxification with a resistance mechanism, identification of specific detoxification enzymes must be accomplished by other means. Subcellular fractions (eg. cytosol and microsomes) prepared from homogenates of insect tissues can be used to measure activities toward insecticide substrates in the presence and absence of appropriate cofactors such as reduced glutathione for glutathione transferase and reduced nicotinamide adenine dinucleotide phosphate (NADPH) for cytochrome P450 dependent monooxygenase.

For the Dursban-R strain (*26*), determining the role of metabolism in chlorpyrifos resistance is complicated by the presence of both monooxygenase-dependent activation of the phosphorothioate molecule to the active acetylcholinesterase inhibitor, chlorpyrifos oxon, and monooxygenase-dependent detoxification. Microsomal metabolism of chlorpyrifos in both strains is dependent on the presence of NADPH in the incubation mixture, indicating involvement of the cytochrome P450 monooxygenase system. Additionally, NADPH-dependent microsomal metabolism was enhanced in the resistant strain, and although this oxidative activity resulted in increased levels of non-toxic metabolites, formation of chlorpyrifos oxon was also enhanced. The increased rate of oxidation and insecticide activation was apparently offset by subsequent hydrolytic metabolism of chlorpyrifos oxon. In assays of cytosolic preparations with both chlorpyrifos and chlorpyrifos oxon as substrates, hydrolytic activity was detected only when chlorpyrifos oxon was used as substrate and was elevated in the resistant strain. The addition of glutathione to the incubations increased formation of aqueous metabolites and substrate depletion in both strains, but the level of differences between strains was similar in the presence and absence of GSH indicating that glutathione transferases are not involved in the resistance. A summary of the metabolic pathways involved in resistance of the Dursban-R strain appear in Figure 3.

*In vitro* metabolism studies of the Baygon-R strain (*31*) also conclusively demonstrated the involvement of both cytochrome P450-dependent monooxygenases and hydrolytic enzymes. *In vitro* microsomal metabolism of $^{14}C$ propoxur in resistant and susceptible strains was again NADPH-dependent and formation of oxidative metabolites was significantly enhanced in the resistant strain. Differences between strains were also noted in cytosolic metabolism of propoxur based on increased loss of substrate and reduced total recovery of substrate and metabolites in the resistant strain. As noted earlier, propoxur hydrolysis eventually gives rise to $CO_2$, and the reduced rates of recovery noted for cytosolic incubations are therefore indicative of hydrolytic propoxur metabolism. Addition of reduced glutathione to the incubation mixture had no observable effect on propoxur metabolism. A summary of the metabolic pathways involved in propoxur resistance appear in Figure 4.

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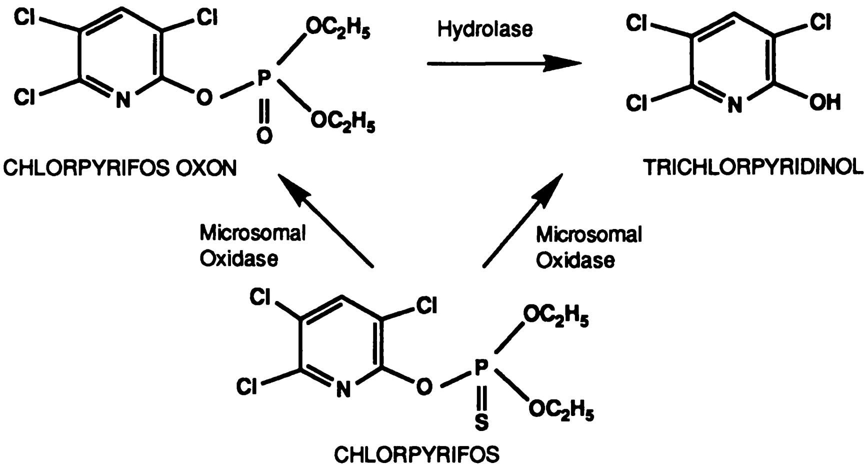
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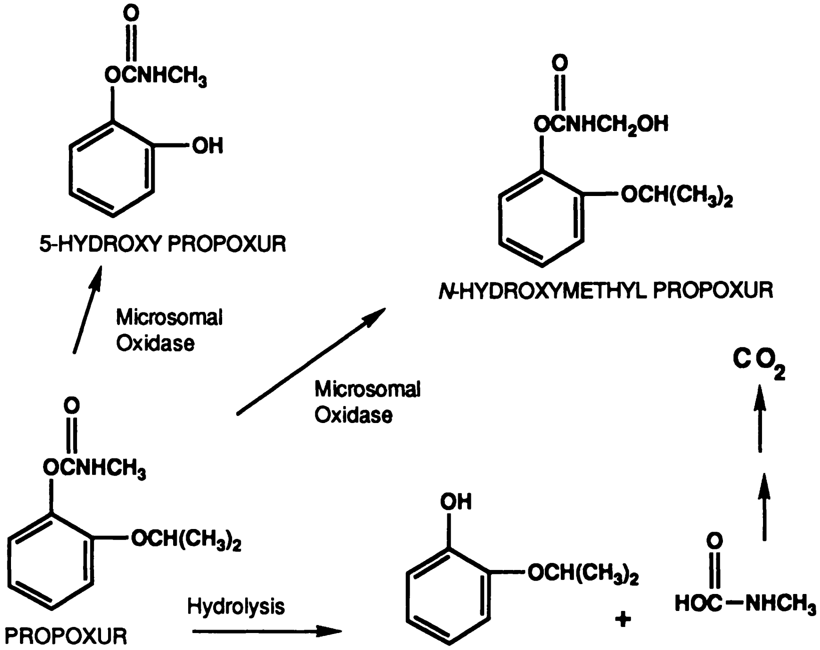
Figure 3. Metabolic mechanisms of chlorpyrifos resistance in the Dursban-R strain.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Microsomal Oxidase
Microsomal Oxidase
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O
$OCNHCH_3$
$OCH(CH_3)_2$
PROPOXUR
Hydrolysis
OH
$OCH(CH_3)_2$
+
O
HOC—$NHCH_3$

Figure 4. Metabolic mechanisms of propoxur resistance in the Baygon-R strain.

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**Metabolic Detoxification and Cross Resistance.** Cross resistance is the phenomenon where a given resistance mechanism confers resistance to more than one insecticidal compound. In German cockroaches, cross resistance has been reported in several instances. Collins (*32*) reported that German cockroaches selected in the laboratory for resistance to diazinon also had significant levels of resistance to malathion, DDT, propoxur, and pyrethrins. Furthermore, selection with propoxur resulted in the development of high levels of resistance to other carbamates, OPs, DDT, and pyrethrins (*41,42*). Multiresistance, or the presence of multiple resistance mechanisms within the same strain, also seems to be relatively common in this species since resistance across insecticide class is often observed in field populations of German cockroaches (*2,24*).

Based on similarities in resistance mechanisms and multiresistance to different insecticide classes, the Dursban-R and Baygon-R strains may have identical metabolic enzyme systems that have enhanced activity toward different insecticide substrates. This possibility was examined by comparing properties of oxidative and hydrolytic enzyme systems in resistant, susceptible and $F_1$ progeny of resistant and susceptible crosses (B.D.S. and J.G.S unplished data). Previous studies have indicated that propoxur resistance in the Baygon-R strain is incompletely recessive and chlorpyrifos resistance in Dursban-R is incompletely dominant (*26,31*). The inheritance of resistance in both strains is somewhat intermediate and both resistance mechanisms are due to elevation of one or more hydrolases plus one or more oxidative enzymes. The R x S hybrids were included in the study to identifiy characteristics of the oxidative and hydrolytic enzyme systems that are related to the resistance, since any trait that is expressed at a similar or lower level than found in the resistant parent could be implicated in the resistance mechanisms.

The combined results from assay of hydrolytic and oxidative enzymes for resistant and susceptible cockroaches indicated that different forms of oxidative and hydrolytic enzymes were responsible for chlorpyrifos and propoxur resistance in these strains. Assays of microsomal monooxygenase components and activities toward model substrates indicated that for the Baygon-R strain, total P450 is elevated relative to a susceptible strain but not the other components of the system (i.e. cytochrome $b_5$ and NADPH cytochrome *c* reductase). Significantly higher oxidative activity was also noted for a series of model substrates in this strain. In contrast, the Dursban-R strain displayed no significant differences from the susceptible strain in any of the assays conducted to assess monooxygenase activity. It is possible that monooxygenase-mediated resistance in this strain is due to elevated levels of a minor P450 isozyme, and therefore, an increase in total P450 would be relatively small and undetected (*43,44*). Regardless of the nature of the modification, it is seems likely that the two strains differ in the nature of oxidative system that confers resistance. The same is true for hydrolytic enzymes in the two strains. Both exhibited elevated activity toward a series of model substrates, but the R x S hybrids displayed striking differences suggesting differences between the two strains: the Dursban-R x susceptible hybrid was not different from the parental susceptible strain and the Baygon-R x susceptible hybrid displayed higher activity than either of the parental strains.

## Conclusions

It is apparent from the preceeding discussion that German cockroaches possess a variety of mechanisms to resist the toxic effects of chemical control agents, and that broad generalizations regarding resistance mechanisms in this species should be avoided. Despite this diversity, certain patterns are beginning to emerge that may impact control strategies and contribute to the development of resistance management programs designed to prevent or at least delay the onset of resistance development.

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Pyrethroid resistance in German cockroaches involves a modification of the central nervous system or *kdr* resistance, and therefore, cross resistance between different pyrethroid insecticides is likely. Additionally, evidence is mounting for the involvement of metabolic detoxification that in combination with *kdr* may confer extremely high levels of pyrethroid resistance. The presence of both target site insensitivity and metabolic resistance may complicate management programs if compounds other than pyrethroids, such as OPs and carbamates, are affected by the metabolic resistance. Cross resistance to newer compounds marketed for German cockroach control, such as hydromethylnon, has not been demonstrated (*33,34*) and therefore, these new compounds provide an alternative to conventional insecticides that can be integrated with pyrethroids into resistance management programs.

With regard to the acetylcholinesterase inhibitors, resistance mechanisms seem to involve both hydrolytic and oxidative metabolic pathways, and in some instances, the two enzyme systems act in concert to confer resistance. The nature of resistance to OP and carbamate insecticides is complicated by the presence of penetration barriers in certain strains that retard movement of the insecticide through the integument. Finally, it seems likely that different forms of metabolic enzymes may evolve from insecticide selection pressure so that identification of a metabolic pathway for a resistance mechanism does not necessarily identify the molecular form of the enzyme that confers resistance. Such differences would make monitoring programs based on biochemical or immunological techniques impractical. However, if metabolic enzymes involved in resistance are specific for a certain insecticide, cross resistance should be minimal. Therefore, resistance management practices based on rotations or alternations among classes of compounds could be successful in reducing the extent of the resistance problem (*45*).

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## Chapter 19

# Insect Resistance to Benzoylphenylureas and Other Insect Growth Regulators

### Mechanisms and Countermeasures

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Chlorfluazuron, teflubenzuron and hexaflumuron are considerably more toxic than diflubenzuron due to their higher retention in the insect. A rapid elimination of diflubenzuron as compared with chlorfluazuron was observed in various insects. The addition of esterase inhibitors to the diet increased the retention and toxicity of diflubenzuron in Tribolium castaneum and Spodoptera littoralis. While hydrolases play an important role in benzoylphenyl urea detoxification and resistance in some insects, oxidases are the major biochemical sites for metabolic resistance in others.

Culex pipiens selected for methoprene showed cross-resistance to other juvenoids but not to diflubenzuron, OPs or pyrethroids. On the other hand, multiresistant strains were somewhat cross-tolerant to Juvenile hormone analogues. The mechanism of resistance seems to be due to increased levels of detoxifying enzymes, decreased penetration and enhanced excretion of the compound. Cross-resistance between cyromazine and diflubenzuron was observed in house fly strains; the mechanism seems target site related more than metabolic.

Insecticide-resistance management programs are needed to preserve these selective IGRs for the benefit of agriculture.

Benzoylphenyl ureas (BPUs) (Figure 1) are selective insecticides acting on insects of various orders by inhibiting chitin formation (1,2), thereby causing abnormal endocuticular deposition and abortive molting (3). Diflubenzuron, the most thoroughly investigated compound of this type, affects the larval stage (4). It acts mainly by ingestion, but in some species it suppresses fecundity (5,6) and

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exhibits ovicidal and contact toxicity (7-10). The main uses of diflubenzuron are against pests in forestry, horticultural and field crops, and in the home (11). The search for more potent acylureas has led to the development of new compounds such as chlorfluazuron (12), teflubenzuron (13) and hexaflumuron (14). The recent BPUs are very potent against important lepidopterous pests, and all three of them were registered recently in Israel for use against the Egyptian cotton leafworm *Spodoptera littoralis* in cotton and ornamentals whereas teflubenzuron is used against the grapevine moths *Lobesia botrana* and *Cryptoblabes gnidiella* in vineyards.

BPUs affect the larval stages which are actively synthesizing chitin. Hence, the adults of nontarget species, *e.g.* parasites and predators, are seldom affected. Parasites of the house fly *Musca domestica* and the gypsy moth *Lymantria dispar* are not affected by diflubenzuron (15-17). In some cases parasite larvae inside treated hosts are sensitive to diflubenzuron but the adults are not affected (18, 19). Predatory mites and adult predators are not appreciably affected when fed on treated host larvae (18,20,21). Hence BPUs are considered important components in integrated pest management (IPM) programs.

Parallel to the emergence of BPUs, a novel chitin synthesis inhibitor, buprofezin (Applaud), has been developed (22,23) (Figure 2). It acts specifically on certain homopteran pests such as the greenhouse whitefly *Trialeurodes vaporariorum* (24,25), the sweetpotato whitefly *Bemisia tabaci* (26-29), the brown planthopper *Nilaparvata lugens* (23,30,31), and the citrus scales *Aonidiella aurantii*,

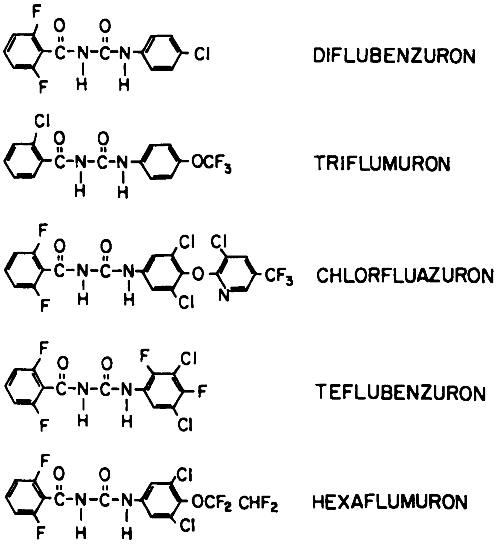

Figure 1. Benzoylphenyl urea insecticides

Saissetia oleae, Icerya purchasi and Planococcus citri (27,32,33). Buprofezin is harmless to aphelinid parasites such as Encarsia formosa and Cales noaki (34-36), to scale parasites such as Aphitis spp. and Comperiella biphasiata (37), and to predacious mites (38), and as such is considered a selective insecticide.

Other insect growth regulators (IGRs) of agricultural and veterinary importance are the triazine compound cyromazine and the juvenile hormone (JH) analogs methoprene, fenoxycarb and pyriproxyfen (Figure 2). Cyromazine, extremely effective against dipteran species, acts at the apolytic stage, thereby affecting the ecdysis process, but has no effect on chitin biosynthesis (39,40). Methoprene is used against stored product pests, house flies and mosquitoes (41,42). Fenoxycarb and pyriproxyfen act specifically against scale insects, affecting egg hatch and adult formation (43-47).

The diverse groups of selective IGRs available today are important components in IPM programs for various agricultural crops. As such they are of utmost significance for future crop protection. Research studies involving mechanisms of resistance should be carried out and resistance management programs should be established in order to prevent development of resistance to these novel insecticides.

This report presents available information on mechanisms of resistance to BPUs and other IGRs along with resistance resurgence and countermeasures.

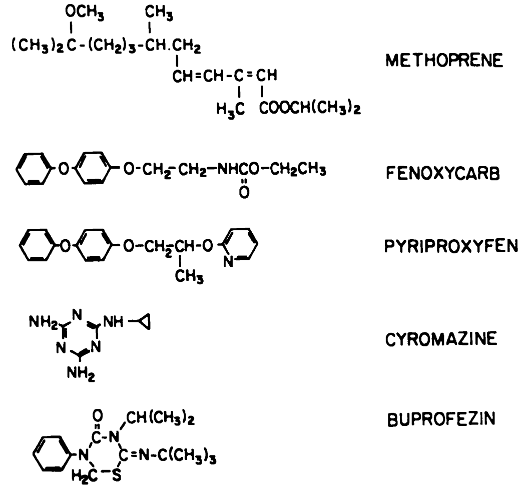

Figure 2. IGRs other than benzoylphenyl ureas

## Mechanisms of Resistance to Benzoylphenyl Ureas

Insect resistance to insecticides can be attained in some cases by target site insensitivity such as in modification of the sodium channels of the nerve axon for DDT and pyrethroids, and in structural alteration of the enzyme acetylcholine esterase for organophosphorus (OP) and carbamate insecticides (48). Decreased

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penetration of insecticides into the insect is an additional factor contributing to insect resistance. This mechanism is usually of minor importance but can act together with others to further increase the resistance level (49). One of the most important factors of insect resistance is probably the increase in metabolic processes leading to high detoxification of the insecticide by enzymes such as carboxylesterases, microsomal oxidases, glutathione transferases and epoxide hydrolases. These processes appear to be controlled in house fly by genes on chromosome II and it is thought that this may be a common codominant resistance gene regulating a variety of detoxifying enzymes (49). Metabolic resistance involves most groups of insecticides such as chlorinated hydrocarbons (48,50,51), OPs (51-53) and pyrethroids (54-58). Multiple resistance combining target site and metabolic mechanisms has developed in various agricultural pests, resulting in control failures (48).

Hydrolysis has been identified as probably the primary route of diflubenzuron detoxification (59). The major hydrolytic metabolites of diflubenzuron in *Spodoptera* species are 4-chloroaniline and 4-chlorophenylurea (60-62). *Spodoptera littoralis* larvae collected from a cotton field in the Jordan Valley of Israel exhibited high resistance to OPs and pyrethroids and mild cross-resistance to teflubenzuron (63). At the $LC_{50}$, the field strain was 120 and 102 times more resistant than the susceptible laboratory strain to chlorpyriphos and cypermethrin, respectively, and five times more tolerant to teflubenzuron (Table I). These results indicate that multiresistance factors caused by various groups of insecticides may confer some cross-resistance to BPUs. The esterase inhibitor TBPT (S,S,S-tributyl phosphorotrithioate) synergized the toxicity of teflubenzuron against the resistant field strain and rendered the larvae again susceptible to teflubenzuron (63). Thus, inhibitors of esterase activity may help, in some cases, to overcome resistance which is due to increased hydrolysis. Assays using radiolabeled diflubenzuron and chlorfluazuron applied to fourth-instar *Tribolium* larvae, revealed a rapid elimination of diflubenzuron (T/2 ~ 7 h) as compared with chlorfluazuron (T/2 > 100 h). Similar differences between the retention of diflubenzuron and chlorfluazuron or teflubenzuron were observed in the Egyptian cotton leafworm *S. littoralis* (61,64,65). The much longer retention of chlorfluazuron in the larvae resulted in over 100-fold increase in toxicity as compared with diflubenzuron (65). Addition of TBPT to the diet increased considerably the retention of diflubenzuron in *Tribolium* larvae (Table II). Furthermore, addition of sublethal dosages of TBPT and profenofos to the diet increased considerably the toxicity of diflubenzuron either in *T. castaneum* (66) or in *S. littoralis* (60), probably due to inhibition of diflubenzuron hydrolase activity (60,66). Under optimal enzyme assay conditions, $10^{-5}$ M profenofos and TBPT inhibited almost completely the *in vitro* degradation diflubenzuron by *S. littoralis* larval gut enzyme (Table III). On the other hand, the more stable BPUs such as chlorfluazuron and teflubenzuron were synergized by esterase inhibitors to a much lesser extent than diflubenzuron and were more resistant to metabolism than diflubenzuron (66).

The relatively low toxicity of diflubenzuron against *Spodoptera exigua* resulted probably from a high detoxification level (62). Addition of TBPT and profenofos to the diflubenzuron treatment enhanced its toxicity nine- and sixfold, respectively. Diethylmaleate, a glutathione transferase inhibitor, decreased the $LC_{50}$ value fivefold, whereas piperonyl butoxide, an oxidase inhibitor, was responsible for only a threefold reduction. Thus, hydrolysis seems to be the predominant route for diflubenzuron detoxification in S. exigua (67).

**Table I. Toxicity of chlorpyriphos, cypermethrin and teflubenzuron for laboratory (S) and field (R) strains of Spodoptera littoralis**

| Compound | Strain | $LC_{50}$, % a.i. | R/S ratio |
|---|---|---|---|
| Chlorpyriphos | S | $1.5\text{x}10^{-4}$ | 120 |
| | R | $1.8\text{x}10^{-2}$ | |
| Cypermethrin | S | $6.1\text{x}10^{-5}$ | 102 |
| | R | $6.2\text{x}10^{-3}$ | |
| Teflubenzuron | S | $3.0\text{x}10^{-5}$ | 5 |
| | R | $1.6\text{x}10^{-4}$ | |

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**Table II. Uptake and retention of radiolabeled [$^{14}$C] diflubenzuron by fourth-instar Tribolium castaneum larvae exposed for 18 h to a diet containing diflubenzuron alone or combined with 100 ppm TBPT or piperonyl butoxide (PB)**

| Compounds | cpm/mg ± SE | | % Retention |
|---|---|---|---|
| | Added to the diet (y) | Found in larvae (x) | [(x+y)100] |
| diflubenzuron | 368±8 | 79±1 | 21 |
| diflubenzuron + TBPT | 325±3 | 134±8 | 41 |
| diflubenzuron + PB | 295±12 | 65±5 | 22 |

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**Table III. Effect of profenofos and TBPT on Spodoptera littoralis diflubenzuron hydrolase activity**

| Inhibitor | concentration (M) | % diflubenzuron hydrolysis relative to control (%) |
|---|---|---|
| Profenofos | $4x10^{-7}$ | 92 |
| | $1.6x10^{-6}$ | 28 |
| | $1.0x10^{-5}$ | 0 |
| TBPT | $4x10^{-7}$ | 42 |
| | $1.6x10^{-6}$ | 32 |
| | $1.0x10^{-5}$ | 3 |

Inhibitors were incubated with the enzyme for 20 min at 37°C prior to enzyme reaction. Enzyme activity is expressed as diflubenzuron hydrolysis relative to control.

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The degradative pathway of diflubenzuron in a laboratory model ecosystem by algae, snails, caterpillars and mosquito larvae is almost entirely through cleavage between the carbonyl and amide groups of the urea bridge to form hydrolytic products (59). The major metabolites found in soil are 4-chlorophenyl urea and 2,6-difluorobenzoic acid (68). In addition, the aniline or the benzoic ring of diflubenzuron can be hydroxylated to form polar materials which can be conjugated and rapidly eliminated from the body (69-72).

While hydrolases play an important role in BPU detoxification and resistance in some agricultural insects, microsomal oxidases are probably of greater importance in the metabolic resistance of other insect species such as the house fly M. domestica (69,73), the boll weevil Anthonomus grandis (70) and the diamondback moth Plutella xylostella (74). Oxidase inhibitors such as piperonyl butoxide and sesamex markedly synergized diflubenzuron in a diflubenzuron selected M. domestica strain (over 1000-fold resistance to diflubenzuron) (73), indicating the key role of oxidases in diflubenzuron resistance in this house fly strain. Limited synergism by TBPT and diethylmaleate demonstrated that esterases and glutathione transferases play a relatively minor role in house fly resistance to diflubenzuron (73). Similarly, piperonyl butoxide decreased the $LC_{50}$ value of the ovo-larvicidal activity of a diflubenzuron-resistant field strain of the apple leaf miner Leucoptera scitella from >6000 mg diflubenzuron/liter to <30 mg diflubenzuron/liter and increased considerably the susceptibility of a diflubenzuron-resistant strain of the spotted tentiform leafminer Phyllonorycter blancardella to diflubenzuron (11).

It may be concluded that the predominant pathway for metabolic resistance to BPUs, whether hydrolytic or oxidative, depends largely

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on the insect species and, to some extent, on the insecticide involved.

Decreased penetration is an additional factor of BPU resistance, affecting specifically ovicidal and trans-ovarial activity. This was apparent in house flies selected to either diflubenzuron or conventional insecticides (75). No information is available yet concerning target site insensitivity in BPU-resistant strains as a possible factor of resistance, probably because of lack of knowledge on the primary biochemical lesion of BPUs in insects which leads to inhibition of chitin formation (76).

**Cross Resistance Between BPUs and Other Insecticides**

Extensive studies have been conducted on cross-resistance between various insecticides and diflubenzuron. In some cases cross-resistance was relatively low or absent, while in others it was conspicuous (11). No appreciable cross-resistance to diflubenzuron was found in a methoprene-resistant strain of Culex quinquefasciatus (77), a DDT-resistant strain of Anopheles quadrimaculatus, a malathion--resistant strain of Aedes taeniorhynchus (42), a chlorpyrifos--resistant strain of C. pipiens (78), and strains of M. domestica with high multi-resistance to OPs (79), carbamates or pyrethroids (80). On the other hand, Rupes et al. (81) and Primprikar and Georghiou (73) reported a strong cross-resistance in house fly larvae between OPs and diflubenzuron. The diflubenzuron resistance level was >1000-fold by topical application, and 146-fold by ingestion when diflubenzuron was mixed with the medium. Cross-resistance between diflubenzuron and cyromazine was reported for larvae of M. domestica in a strain from Texas which had shown control failure with cyromazine (82). In this case, the resistance ratios to cyromazine and diflubenzuron were, respectively, 6.5 and 10.0 at the $LC_{50}$ and 6.2 and 7.6 at the $LC_{95}$ levels. In other studies, the resistance ratios of a cyromazine-resistant housefly obtained from Beltsville, MD, were 105 and 11 at the $LC_{50}$s and 46 and 5 at the $LC_{90}$s for cyromazine and diflubenzuron, respectively (11). Genetic studies showed that the same, or a closely linked, gene conferred resistance to the two compounds (82).

Diflubenzuron larvicidal and transovarial-ovicidal toxicities are not necessarily affected similarly by cross-resistance. A considerable discrepancy in cross-resistance for a field-collected OP-resistant strain of M. domestica was reported by Grosscurt (79). In this case, cross-resistance to diflubenzuron was relatively low (R/S < 5) for larvicidal activity and very high (R/S = 88) for ovicidal activity. These findings could result from different mechanisms. Ingestion of diflubenzuron, which is the main factor for the larvicidal activity, is prone to metabolic resistance, i.e., increase in detoxifying enzymes. On the other hand, the transovarial-ovicidal resistance may result from decreased penetration of diflubenzuron through the female cuticle or the egg shell.

In diet feeding assays carried out with T. castaneum (76,83), hexaflumuron, teflubenzuron and chlorfluazuron were 4- to 23-fold more toxic than diflubenzuron and exhibited similar toxicity on both malathion-susceptible (bb) and -resistant (CTC-12) strains. On the other hand, diflubenzuron was considerably less toxic to the resistant strain, which seems to be due to diflubenzuron's susceptibility to the relatively high oxidative and hydrolytic activities present in this strain (84). A field strain of S. littoralis, which was over 100-fold resistant to pyrethroids and OPs, showed a mild cross- resistance (~fivefold) to teflubenzuron (Table I). Perng and Sun (85) reported that a resistant diamondback moth P. xylostella showed negligible (two- to threefold) cross-resistance to teflubenzuron with no effect on chlorfluazuron. Similarly, a strain of S. littoralis exhibiting 146-fold resistance to monocrotophos, was found to be ~3-fold more tolerant to diflubenzuron and slightly more susceptible to chlorfluazuron when compared with a susceptible laboratory strain (86). In some cases diflubenzuron showed greater potency on a specifically malathion-resistant strain of T. castaneum (Kuala Lumpur strain) than on a susceptible laboratory strain (87).

Due to relatively low or no cross-resistance between conventional insecticides and BPUs in some insect species, BPUs may be used to relax selection pressure to OPs and pyrethroids and, as such, should be considered as important components for the management of insecticide resistance. In addition, the high toxicity of some BPUs against agricultural insect pests and their relatively low toxicity to man and natural enemies render these compounds important tools in IPM programs.

## Development of Resistance to BPUs and Counter Strategies

The incidence of resistance to most of the conventional insecticides has increased substantially in recent years, especially among arthropods of agricultural importance (48,88). Furthermore, laboratory and field studies have demonstrated that resistance can develop toward pesticides with a novel mode of action such as the JHA methoprene (89,90), the triazine compound cyromazine (82,91,92), and the BPUs diflubenzuron, triflumuron and chlorfluazuron (74,75,93).

Several reports indicate high selection for resistance to BPUs in general and to diflubenzuron in particular. A R/S ratio of 50 to diflubenzuron was observed in M. domestica after ten generations of diflubenzuron selection (75). A more severe resistance of 291-fold to diflubenzuron was observed in S. littoralis after 30 generations of selection using topical application (94). Following spraying the manure for several years in the Dutch province of Limburg with diflubenzuron, the R/S ratio of house flies collected from this area vs the susceptible Boeksteyn strain was 103 (11). On the other hand, incorporation of diflubenzuron into the bolus which gradually releases the compound into the digestive tract of the cattle in a quantity sufficient to control stable and face flies in the manure, was a more favorable than other fly control measures in preventing development of resistance (11). An insecticide resistance management (IRM) program restricting the use of diflubenzuron-feeding treatment to 6 weeks with no more than two treatments per year resulted in an unchanged susceptibility during the two-year testing period (11). Hence, a carefully considered IRM strategy is of importance in preventing development of resistance to the novel IGR compounds.

Very pronounced resistance to diflubenzuron was detected in two lepidopterous pests, the apple leaf blotch miner L. scitella and the spotted tentiform leafminer P. blancardella, both of which are severe pests of apples in the Ferrara region in Italy where diflubenzuron has been used heavily since 1977 for controlling these pests. Based on

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$LC_{90}$ values, the Ferrara strain of L. scitella showed a resistance ratio of >7500, while a field strain of P. blancardella could not be killed at a concentration as high as 5000 mg diflubenzuron/liter. A 1:5 diflubenzuron:piperonyl butoxide combination reduced the $LC_{50}$ for both species from >6000 mg diflubenzuron/liter to approximately 30 mg diflubenzuron/liter (11). Of special interest is the recent use of IGRs for controlling the diamondback moth P. xylostella, a very serious pest of cruciferous plants in southeast Asia. In both Malaysia and Thailand, relatively strong resistance to BPUs and control failures were observed in many districts after 2 years of use. The diamondback moth showed resistance to teflubenzuron and chlorfluazuron of 12-16-fold and 16-18-fold, respectively (93).

Countermeasures to overcome development of resistance to BPUs should include restriction to one treatment per a season. Rotation with other insecticides should involve compounds with a different mode of action and those exhibiting no cross-resistance to BPUs. Such a strategy was successful in maintaining susceptibility of manure flies to diflubenzuron during two-year treatment period (11). In some cases, addition of synergists as inhibitors of detoxifying enzymes may largely overcome the resistance due to increased metabolism. Addition of the esterase inhibitor TBPT increased the susceptibility of an OP- and pyrethroid-resistant strain of S. littoralis to teflubenzuron (63), and TBPT and profenofos the susceptibility of T. castaneum, Platynota stultana and Spodoptera species to diflubenzuron (60,66,95). In other cases, the oxidase inhibitors piperonyl butoxide and sesamex decreased considerably the resistance to diflubenzuron in various insect species such as the apple leaf blotch miner L. scitella, the spotted tentiform leafminer P. blancardella and the house fly M. domestica (11,73).

In addition to BPUs, other selective insecticides are available today, such as the JH mimics fenoxycarb and pyriproxyfen and the triazine compound cyromazine. The diversity of selective compounds acting on various biochemical sites in insects enables the buildup of IRM strategies useful in IPM programs for various agricultural crops.

## IGRs Other than Benzoylphenyl Ureas

Juvenoids and especially methoprene (Figure 2) have long been used for controlling mosquito larvae (96-100) and stored-product pests (101). The most applaudable finding was that methoprene is equally efficient in controlling susceptible strains of mosquitoes and those resistant to conventional insecticides (42). On the other hand, multiresistant strains of T. castaneum (102), Heliothis virescens (103) and M. domestica (104) were somewhat cross-resistant to Juvenile hormone analogues. Methoprene selection for 12 generations to a multiresistant strain of Culex tarsalis raised its existing tenfold cross-resistance to x90 (105). Methoprene selection of a susceptible strain of C. pipiens induced 13-fold resistance in eight generations (106). The methoprene resistance which developed in C. pipiens was at first deleterious to the insect, resulting in a strong suppression of the reproductive system. However, after 40 generations of selection pressure and the attainment of a 100-fold resistance, the reproductive capacity returned to normal. These insects showed cross-resistance to other JH mimics but not to diflubenzuron or to conventional insecticides (89). S. littoralis selected to diflubenzuron (290-fold

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resistance after 30 generations) exhibited a clear case of negative correlation to resistance when challenged with methoprene or other JH analogs (94). Similarly, Brown et al. (89) found that resistance to methoprene in C. pipiens showed no cross-resistance to either diflubenzuron or conventional insecticides.

Comprehensive studies of JHs and juvenoids' metabolism have been reported (107-109). JHs are metabolized in insects by esterases and oxidases (110). Similar degradative pathways have been reported for various JHAs (111-113). It has been generally accepted that epoxide hydration and ester cleavage are the great primary routes of JH metabolism. Ester cleavage is apparently of major importance in lepidopteran insects and epoxide hydration in dipteran species (109). In general, oxidative metabolism appears to be minor when compared with hydrolytic pathways. However, some resistant strains of house fly metabolize JH rapidly through oxidation (114,115).

Although ester cleavage was reported as an important metabolic pathway in several dipteran species for dienoate juvenoids (116-118), the isopropyl ester of methoprene has generally been refractory to esterases (109,116-118). In vivo ester cleavage of methoprene has been reported for Tenebrio molitor, Oncopeltus fasciatus, M. domestica, Aedes aegypti and C. quinquefasciatus (119,120). In some cases, oxidative pathways are important in the metabolism of methoprene. Solomon and Metcalf (119) demonstrated that methoprene was converted to $CO_2$, and oxidative metabolites were produced in vivo in both T. molitor and O. fasciatus. In addition, oxidative O-demethylation is important in methoprene metabolism in various insects (114,119-121). The presence of another oxidative pathway such as epoxidation has been shown with hydropene and methoprene in several species of Diptera (114,117).

The mechanism of methoprene resistance in C. pipiens has been examined in larvae selected with methoprene for over 30 generations; the resistance level was found to reach approximately 200-fold (121). The amount of methoprene which penetrated these larvae was very low and most of the identified materials were in the form of polar conjugates (121). The major metabolite was the hydroxy-ester, and piperonyl butoxide reduced the resistance ratio from 213 to 136. Thus, oxidative metabolism appeared to play an important role in methoprene resistance in this pest. In addition, the resistance was associated with faster excretion, poorer distribution in the tissues and, possibly, reduced intake (122). In methoprene-selected house flies, the primary metabolite both in vitro and in vivo was the 11-hydroxy compound resulting from O-demethylation (90-117). Other reports indicate that tissues such as the imaginal disks exhibit high levels of JH esterase and epoxide hydrolase (123-125), which could play a role in the degradation of methoprene.

Other juvenoids of agricultural importance are fenoxycarb and its derivative pyriproxyfen. Pyriproxyfen, which proved to be more potent than fenoxycarb on various insect species, was introduced recently for controlling whiteflies in cotton (43,126) and scale insects in citrus (47). With the latter juvenoids, no established information on resistance development and mechanisms is yet available.

Other IGRs which have been introduced recently for controlling agricultural pests are the triazine compound cyromazine and the chitin synthesis inhibitor buprofezin (Figure 2). Cyromazine, representing a new class of IGRs, acts selectively against a number of dipterous

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species (127-132). In some cases, it controls species other than dipterans, such as the fall armyworm Spodoptera frugiperda (133), the tobacco hornworm Manduca sexta (134) and the dog flea Ctenocephalides canis (135). Laboratory selection of house flies with cyromazine for 15 generations increased resistance ca 70-fold compared with a susceptible strain (92). A pronounced cross-resistance between cyromazine and diflubenzuron in field house fly strains was observed in at least two locations in the US (11,82). On the other hand, no cross-resistance to cyromazine could be detected in several house fly strains resistant to other insecticides (82,136). The resistance mechanism is probably not due to an enhanced level of MFOs, since piperonyl butoxide did not synergize the toxicity of cyromazine on either the cyromazine-resistant or -susceptible house fly strain (82,136). In addition, diethylmaleate, the glutathione-transferase inhibitor, did not affect cyromazine toxicity (136). Thus, the resistance gene seems to be associated with the biochemical target site of the insecticide rather than with its metabolic pathway (82).

Buprofezin, a novel IGR compound, inhibits chitin synthesis specifically in some sucking insects such as whiteflies, plant hoppers and scale insects (76). It was introduced recently for controlling the sweetpotato whitefly B. tabaci in cotton fields in Israel (26,29). Two years of buprofezin application carried out according to an IRM strategy (the use of buprofezin was restricted to one or two applications per season) did not affect the performance of the IGR for controlling B. tabaci (137). In addition, a field strain of B. tabaci six- to sevenfold more resistant to endosulfan showed no cross-resistance to buprofezin (137).

Intensive studies of resistance, in general, and of the resistance mechanisms, in particular, for the various novel IGRs are of utmost need for establishing IRM stategies aiming at preventing resistance development and preserving these selective compounds for the benefit of agriculture.

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## Chapter 20

# Mechanisms of Abamectin Resistance in the Colorado Potato Beetle

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Two abamectin-resistant strains of Colorado potato beetle have been produced by field and ethyl methanesulfonate selection schemes. Multiple backcrossings resulted in isogenic strains which are 23- and 15-fold resistant, respectively, but show no cross-resistance to dieldrin, azinphosmethyl or permethrin. Resistance is autosomal, incompletely recessive, and polyfactorial for both strains. High levels of oxidative synergism, elevated cytochrome P450 levels and increased amounts of oxidative metabolites of $[^3H]$avermectin B1a substantiates a monooxygenase-based resistance. Additionally, esteratic synergism and elevated hydrolytic activity indicates a carboxylesterase-based resistance. The lack of hydrolytic metabolites of $[^3H]$avermectin B1a, however, suggests a sequestration role for the resistant carboxylesterase. Penetration and excretion factors play no significant role in resistance nor does there appear to be a significant glutathione-S-transferase component.

The avermectins are a group of closely related 16-membered macrocyclic lactones with potent acaricidal, insecticidal and nematicidal activities. They are natural products produced during fermentation by a soil actinomycete microorganism, *Streptomyces avermitilis*. This process results in the production of 4 homologous pairs of highly related compounds: Avermectin $A_1$, $A_2$, $B_1$, $B_2$. Avermectin B1 (abamectin, MK-936) is the major component isolated from the fermentation broth and is a mixture of homologous avermectins containing a minimum of 80% avermectin B1a and a maximum of 20% avermectin B1b (*1*).

Since their discovery in 1976 and subsequent identification as novel pesticides and drugs, the avermectins have had a tremendous impact on veterinary medicine and whose potential in human medicine and insect control is enormous (*2, 3*). Because of this, it is vital that resistance management strategies be devised prior to the appearance of resistance in field situations when it is too late to preserve the inherent susceptibility of the target pest population. The availability of abamectin-resistant insect strains through mutational and genetic selection schemes would greatly assist in the development of such programs. Resistance management strategies (i.e., knowledge of the genetic inheritability of resistance factors, biochemical mechanisms of resistance, effective application schemes, proper use of synergists, identification of compounds with negative cross-resistance, etc.) and

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accurate, sensitive, biochemical monitoring schemes (*4, 5*) could be developed before commercial use of abamectin. The availability of such information and diagnostic techniques could greatly extend the effective life span of these important pest control agents.

In this chapter, we will describe the selection process for abamectin resistance in Colorado potato beetle (CPB), the genetics underlying this resistance and the biochemical mechanisms of resistance.

## Resistance to the Avermectin(s) by Arthropod Pests

Since the discovery of their antihelminthic and insecticidal properties in 1979, the avermectins have been rapidly accepted as highly effective pest control agents. These agents now have widespread use in application strategies to protect agricultural, horticultural and animal commodities (*3*). As intensity of use increases, it is very likely that avermectin resistance will become a commercially significant problem. Because of their growing importance, every means possible should be directed to extending the usefulness of these novel pesticide compounds.

The first report of possible cross-resistance to the avermectins was made by Abro et al. (*6*) concerning a low level of cross-resistance in a multiply-resistant field strain of *Plutella xylostella* from Thialand. This strain of the diamondback moth was 3 to 26-fold resistant to avermactin depending on the application means and/or type of fomulation used. Because no isogenic strains were prepared, it was impossible to rule out the possible role of vigor tolerance in this comparison.

Abamectin cross-resistance was subsequently established in pyrethroid-resistant *Musca domestica* (*7*). Using a laboratory-selected strain (LPR) and a field-collected strain (Dairy) of house fly, a 25-fold and a 5.9-fold level of resistance was established using $LD_{50}$ values for these strains, respectively. The genetics of this cross-resistance was determined to be polygenetic and associated with genes on autosomes 2 and 3. Abamectin cross-resistance in this instance was concluded to be due to decreased cuticular penetration and increased oxidative detoxication. Most recently, laboratory selections have resulted in extremely high levels of abamectin resistance (36 to 60,000-fold) in field-collected house flies (*8*). This particular resistance was not synergised by piperonyl butoxide, S,S,S,-tributyl phosphorotrithioate or diethyl malate. Also, no increases in (cross)-resistance levels to a number of organophosphates, permethrin or cyclodienes were evident after laboratory selections of highly abamectin-resistant strains. Subsequent biochemical and genetic studies on the highly abamectin-resistant AVER strain of house fly has determined this resitance to autosomal, recessive and polyfactorial (*9*). Two biochemical mechanisms have been associated with this resistance; decreased cuticular penetration and altered abamectin binding due to a reduction in the number of specfic binding sites. Interestingly, the AVER strain showed no differences in *in vivo* metabolism compared to susceptible strains.

A low level of abamectin resistance (3.8-fold) has also been selected for in a laboratory strain of the western predatory mite, *Metaseiulus occidentalis*, (*10*). The heterogeneous colony from which the abamectin-resistant strain was established included strains resistant to organophosphates, sulfur, carbaryl and permethrin. Resistance to abamectin occurred gradually over the 20 generations

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of selection with only modest increases in survivorship between selections. More recently, two insecticide-resistant laboratory strains of German cockroaches (*Blattella germanica*) showed significant cross-resistance (10-fold) to abamectin based on $LD_{95}$ values (*11*). Interestingly, the Kenly strain is a multiresistant strain which elicits a piperonyl butoxide (PBO) and S,S,S,-tributyl phosphorotrithioate (DEF) suppressible resistance to propoxur and bendicarb. The PYR strain was selected from the Kenly strain using pyrethrins.

## Selection of Isogenic Strains of CPB Resistant to Abamectin

For our studies, we chose an insect which is one of the most notorious examples of pest control failure due to insecticide resistance, the Colorado potato beetle (CPB; Leptinotarsa decemlineata (Say)). A major problem associated with insecticide resistance management strategies is that the mechanism of resistance is discovered only after field selection has already made the pest population resistant. In an attempt to stem this problem, two abamectin resistant strains of CPB were generated by separate means. The mutagen, ethyl methanesulfonate, in conjunction with selection at a discriminatory dose, was used to select an abamectin-resistant strain from a susceptible laboratory strain (*12, 13*). The other resistant strain was generated through an intense selection with abamectin of a field strain contained in cages set up in existing potato fields (*12*).

Although such selectively induced mutations may not be identical to that which may occur under field conditions, they still provide a useful model for the study of resistance mechanisms, particularly for insecticides that have not had extensive use commercially. Additionally, by comparing the two abamectin resistant strains isolated by these separate techniques, it will be possible to assess the relative merits of using mutagens such as EMS to produce insecticide resistance.

**Field/Laboratory Selection.** For the establishment of an abamectin-resistant CPB strain through field selection studies (i.e., AB-Fd $F_6$), field beetles housed in large cages maintained in established potato fields were sprayed with abamectin every 1 to 2 weeks throughout the growing season at a rate of 4.5 g abamectin 0.4047 $ha^{-1}$ (i.e., $acre^{-1}$), a dose that had been determined to kill 99% of the field CPB population (*12*). Application was made approximately 20 times over a 2 year period. At the end of the second growing season (e.g., 4-6 generations of selection), CPB survivors were taken into the laboratory, dosed at 15 ng $beetle^{-1}$ and those which survived were interbred. These progeny were designated as the AB-Fd $F_1$ strain (Figure 1). This strain was allowed to interbreed an additional six generations in the laboratory. Each generation was selected at the 10 ng $beetle^{-1}$ level or higher until the resistance level stabilized. This abamectin resistant-strain was designated as AB-Fd $F_6$ (Figure 1).

**Ethyl Methylsulfonate Selection.** Newly emerged susceptible laboratory (SS) male CPBs were exposed to EMS in a manner similar to *Musca domestica* (*14*) to create an abamectin-resistant strain (i.e., AB-L $F_7$). Several ml of 0.025 M EMS solution in sterile 1% sucrose solution was applied to a crumpled kimwipe in a 0.473 liter (1 pint) glass jar until saturation. After exposure for 24h, males were washed twice in 70% alcohol to rinse off any excess EMS and placed on potato plants with untreated females. Females were not directly exposed to EMS since this damages oocytes (*13*). A 30% reduction in fertility for these pairs indicated a preferable mutation rate. The $F_1$ larvae were reared to fourth instars and given a discriminatory dose of 15 ng abamectin beetle$^{-1}$. These survivors were mated to susceptible CPBs for two generations to produce a resistant strain (AB-L $F_3$, Figure 2). This resistant strain was interbred for 4 additional generations. At each generation, only those CPBs able to survive a discriminating dose of 10 ng or higher were used to establish the AB-L $F_7$ strain (Figure 2).

## Population Genetics of Abamectin-Resistant Strains of CPB

There was no difference between either AB-Fd $F_6$ reciprocal $F_1$ crosses ($\chi^2$= 9.44; df = 2; $P$ = 0.009, Figure 1) or AB-L $F_7$ reciprocal $F_1$ crosses ($\chi^2$= 3.51; df = 2; $P$ = 0.173, Figure 2), indicating an autosomal, incompletely recessive (0.3 to 0.4 level of dominance, respectively) form of inheritance of abamectin resistance in both strains.

Classic backcrossing techniques were used to determine the number of factors involved in abamectin resistance, although determining the number of factors with this method is difficult when the SS and $F_1$ mortality lines overlap (*15*). The second backcross of the AB-Fd strain (AB-Fd $Bc_2$) showed no significant difference from the predicted monogenic curve ($x^2$=0.91; *df*=2; $P$=0.633) (Figure 3). However, the following backcross generation (AB-Fd $Bc_3$) was significantly different from the predicted monogenic curve, although the probability value was only slightly less than 0.05 ($x^2$=6.30; *df*=2; $P$=0.043), and the AB-Fd $Bc_3$ cross was also significantly different from the AB-Fd $Bc_2$ ($x^2$=14.08; *df*=2; $P$=0.001). These two differences indicate that abamectin resistance is polyfactorial in this strain.

The AB-L $Bc_2$ backcross showed no significant difference from the predicted monogenic curve ($x^2$=3.45; *df*=2; $P$=0.178) (Figure 4). The AB-L $Bc_3$ cross also was not significantly different from the predicted monogenic curve ($x^2$=5.18; *df*=2; $P$=0.075), although the slopes of these two curves were significantly different ($x^2$=4.29; *df*=1; $P$=0.038). The two probability values (i.e., $P$) generated in this comparison were both very close to 0.05, making it difficult to distinguish inheritance as mono- or polyfactorial. However, comparison of the AB-L $Bc_2$ and AB-L $Bc_3$ demonstrated a significantly high level of difference between the two backcrosses ($x^2$=22.94; *df*=2; $P$<0.001), indicating polyfactorial inheritance of abamectin resistance in the AB-L strain as demonstrated in the AB-Fd strain. These abamectin-resistant strains are now approximately 95% isogenic to the susceptible (SS) strain.

**Resistance Levels and Cross-Resistance Patterns.** Abamectin was determined to be a very effective insecticide against both field (MA-R) and susceptible laboratory (SS) strains of CPB (Table I, $LD_{50}$ = 1.95-1.98 ng beetle$^{-1}$, respectively). The MA-R strain, which is highly resistant to organophosphate and pyrethroid insecticides (*16*) was not significantly different in its mortality response to abamectin compared to the SS strain ($x^2$ = 1.57; *df* = 2; $P$ = 0.46). This apparently indicates an absence of cross-resistance to abamectin in the MA-R field strain. A dose of 10 ng abamectin beetle$^{-1}$, which is higher than the $LD_{97}$, was selected as the discriminating dose between susceptible and resistant strains. Both AB-Fd and AB-L strains had little mortality at 10 ng abamectin beetle$^{-1}$, while this dose caused approximately 99% mortality in the SS strain (Figures 3 & 4).

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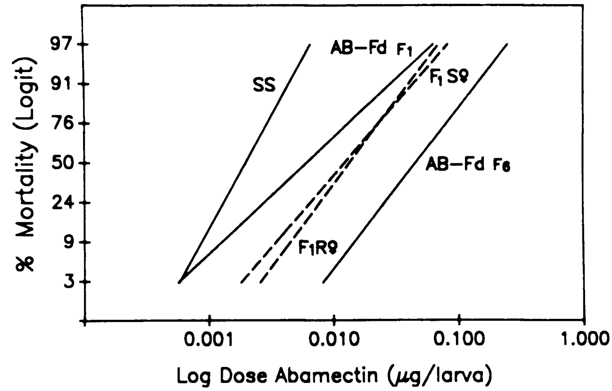

Figure 1. Log-dose versus logit mortality regressions of fourth instar CPB larval of the North Carolina strain (SS), field-selected strain (AB-Fd $F_6$), $F_1$ progeny obtained by crossing an AB-Fd $F_6$ female to an SS male ($F_1$R , dashed line), $F_1$ progeny obtained by crossing an SS female to an AB-Fd $F_6$ male ($F_1$S , dashed line), and the progeny from the first abamectin-resistant generation of CPBs brought into the laboratory from field cages (AB-Fd $F_1$) (Reproduced with permission from ref. 12. Copyright 1990 Society of Chemical Industry.)

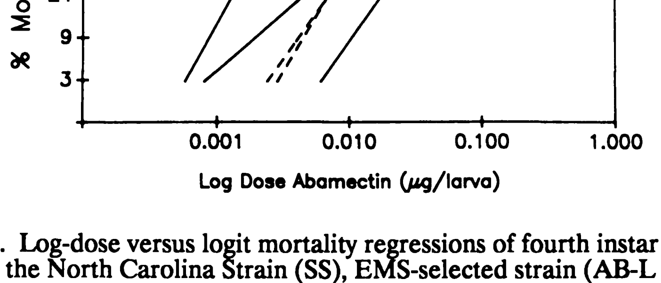

Figure 2. Log-dose versus logit mortality regressions of fourth instar CPB larval of the North Carolina Strain (SS), EMS-selected strain (AB-L $F_7$), $F_1$ progeny obtained by crossing an AB-L $F_7$ female to an SS male ($F_1$R , dashed line), $F_1$ progeny obtained by crossing an SS female to an AB-L $F_7$ male ($F_1$S , dashed line), and the progeny produced by interbreeding the $F_2$ generation of EMS- treated CPBs (AB-L $F_3$) reistant to abamectin. (Reproduced with permission from ref. 12. Copyright 1990 Society of Chemical Industry.)

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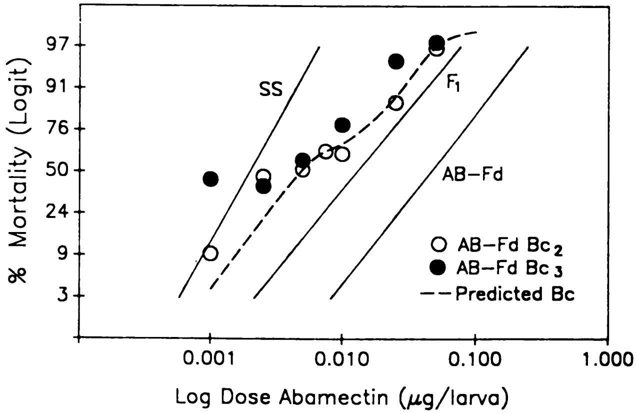

Figure 3. Log-dose versus logit mortality regressions of fourth-instar CPB larvae of the susceptible (SS) and abamectin-resistant strain from field selections (AB-Fd), pooled reciprocal $F_1$ crosses ($F_1$), backcross generations 2 and 3 ($BC_2$ and $BC_3$), and the predicted backcross response for monofactorial inheritance determined as 1/2 $F_1$ mortality + 1/2 SS mortality at any given dose (dashed line).

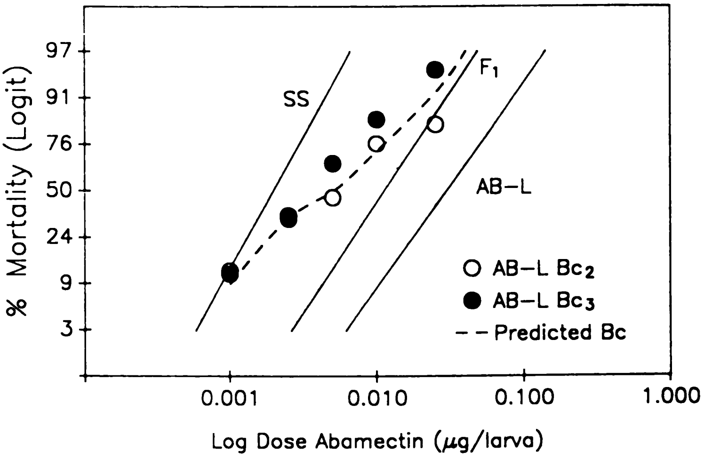

Figure 4. Log-dose versus logit mortality regressions of fourth-instar CPB larvae of the susceptible (SS) and abamectin-resistant strain from EMS selections (AB-L), pooled reciprocal $F_1$ crosses ($F_1$), backcross generations 2 and 3 ($BC_2$ and $BC_3$), and the predicted backcross response for monofactorial inheritance determined as 1/2 $F_1$ mortality + 1/2 SS mortality at any given dose (dashed line).

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Table I. Comparative topical toxicities of fourth-instar larvae of the susceptible (SS), multiply-resistant Massachusetts (MA-R), and abamectin-resistant (AB-Fd & AB-L) strains of CPB to abamectin and dieldrin

| | N | $LD_{50}$(95% CI) | $RR(LD_{50})^a$ | $LD_{97}$(95% CI) | $RR(LD_{97})^a$ | Slope ± SEM |
|---|---|---|---|---|---|---|
| Abamectin<br>(ng/larva) | | | | | | |
| SS | 540 | 1.95(1.47,2.31) | - | 6.62(4.92,12.3) | - | 6.56 ± 0.97 |
| MA-R | 251 | 1.98(1.69,2.39) | - | 4.95(3.61,10.4) | - | 8.76 ± 1.89 |
| AB-Fd $F_1$ | 936 | 12.7(11.5,14.0) | 7 | 39.2(33.7,47.4) | 6 | 4.51 ± 0.32 |
| AB-Fd | 482 | 45.3(32.6,56.2) | 23 | 249.8(145.6,1291) | 38 | 4.68 ± 0.65 |
| AB-L $F_1$ | 1006 | 11.1(10.1,12.2) | 6 | 28.1(24.7,32,9) | 4 | 5.47 ± 0.39 |
| AB-L | 568 | 29.4(23.9,34.4) | 15 | 142.2(112.4,200.9) | 21 | 5.08 ± 0.56 |
| Dieldrin<br>(ug/larva) | | | | | | |
| SS | 219 | 0.72(0.53,0.94) | - | 14.5(7.1,55.5) | 0 | 2.66 ± 0.43 |
| MA-R | 267 | 1.32(0.89,1.85) | 2 | 31.4(15.8,100.1) | 2 | 2.53 ± 0.36 |
| AB-Fd | 256 | 0.52(0.14,15.33) | 1 | 53.2(7.7,7186.2) | 3 | 1.73 ± 0.25 |
| AB-L | 340 | 0.70(0.53,0.89) | 1 | 15.9(9.2,35.9) | 1 | 2.57 ± 0.28 |

[a] Resistance ratio (RR) = $LD_x$ of each strain/$LD_x$ SS strain.

The AB-Fd strain is more resistant (23-fold vs. 15-fold, Table I) and significantly different from the AB-L strain ($\chi^2 = 25.83$; $df = 2$; $P < 0.001$, Table I). The slight difference in the level of resistance of the two strains may be due to differences in vigor tolerance between them since the AB-Fd strain was isolated from a Massachusetts population and the AB-L strain originated from a North Carolina population.

No cross-resistance to dieldrin was evident in the MA-R or the abamectin-resistant strains as measured by resistance ratio (Table I). This does not rule out the possibility of a site-insensitivity resistance mechanism for abamectin, since dieldrin binds to the picrotoxinin site on the GABA-chloride channel (*17*). An alteration in one binding site may or may not have an allosteric effect on any other binding site. So far, no cross-resistance to abamectin has been observed in dieldrin-resistant strains where site-insensitivity has been established as the mechanism of resistance (*18*, *19*). Recently, an abamectin-resistant strain of house fly (AVER) with altered abamectin binding was shown not to be cross-resistant to dieldrin or lindane (8,9). However, chloride-flux studies will definitively demonstrate whether site-insensitivity is a mechanism in abamectin resistance in either of the abamectin-resistant CPB strains. Neither the AB-F nor the AB-L strains showed any cross-resistance to either azinphosmethyl or permethrin (unpublished data, J.A. Argentine).

In Vivo **Synergism.** Piperonyl butoxide (PBO) produced very high levels of synergism to abamectin in both abamectin-resistant strains (Table II). The synergistic ratios (SRs) of the AB-Fd and AB-L strains were 19 and 15, respectively. PBO also synergized abamectintoxicity in the SS strain, but at a much more reduced level relative to the abamectin-resistant strains. This is shown in the high relative percent synergism (R%S) values of the abamectin-resistant strains (68 and 70, AB-Fd and AB-L, respectively, Table II).

The esteratic synergist, DEF (*S*, *S*, *S*-tributyl phosphorotrithioate), produced a moderate level of synergism in both abamectin-resistant strains (SR = 5) and little in the SS strain (SR =2). Although DEF synergism is not of the same magnitude as PBO, this increase in the SR value of the abamectin-resistant strains could possibly indicate esterase involvement in abamectin resistance.

The glutathione-S-transferase inhibitor, DEM (diethyl maleate), had a small but similar affect on abamectin toxicity in both the susceptible and resistant strains (Table II).

## Biochemical Mechanisms of Abamectin Resistance in CPB

**Pharmacokinetics of $[^3H]$Avermectin B1a.** Cuticular penetration of $[^3H]$avermectin B1a was similar for all strains and resulted in only approximately 15-25% of the topically-applied compound remaining on the surface after 6 hr. Although there was a significant difference between the AB-L and SS strain at 6 hr in the amount of compound left on the cuticle, a similar situation was not present in the AB-Fd strain and the difference in the AB-L strain was slight. Thus, it is unlikely that differential penetration is contributing significantly to overall abamectin resistance.

At 6 hr, both the AB-Fd and AB-L strains had significantly lower levels of radioactivity internally and higher levels in the excrement (Table III). This

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**Table II. Effect of metabolic synergists on the toxicity of abamectin in fourth-instar larvae of the susceptible (SS) and abamectin-resistant (AB-Fd & AB-L) strains of CPB**

| | N | $LD_{50}$(95% CI) | Slope(SEM) | SR[a] | R%S[b] |
|---|---|---|---|---|---|
| | | (ng/beetle) | | | |
| SS | 540 | 1.95 (1.47-2.31) | 6.56 (0.97) | - | - |
| DEF | 287 | 0.96 (0.80-1.15) | 3.73 (0.45) | 2 | 21 |
| DEM | 287 | 1.62 (1.08-2.39) | 3.26 (0.37) | 1 | 18 |
| PBO | 288 | 0.61 (0.34-1.01) | 5.37 (0.67) | 3 | 21 |
| AB-Fd | 482 | 45.30(32.60-56.20) | 4.68 (0.65) | - | - |
| DEF | 305 | 8.87 (5.84-11.61) | 5.89 (1.06) | 5 | 42 |
| DEM | 311 | 23.72(19.67-28.68) | 3.42 (0.43) | 2 | 19 |
| PBO | 245 | 2.40 (1.92-2.88) | 4.85 (0.64) | 19 | 68 |
| AB-L | 568 | 29.40(23.90-34.42) | 5.08 (0.56) | - | - |
| DEF | 268 | 5.66 (2.84-7.90) | 4.66 (0.91) | 5 | 48 |
| DEM | 302 | 10.40 (8.74-12.34) | 3.90 (0.43) | 2 | 35 |
| PBO | 237 | 1.94 (1.40-2.62) | 4.84 (0.64) | 15 | 70 |

[a] Synergistic Ratio (SR) = $LD_{50}/sLD_{50}$.

[b] Relative percent synergism of susceptible strain; R%S(S) = 100[log $LD_{50}$(S)-log $sLD_{50}$(S)]/[log $LD_{50}$(R)-log $sLD_{50}$(S); Relative percent synergism of resistant strain; R%S(R) = 100[log $LD_{50}$(R)-log $sLD_{50}$(R)]/[log $LD_{50}$(R)-log $sLD_{50}$(S).

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Table III. Pharmocokinetics of $[^3H]$Avermectin B1a (0.46ng/larva) in fourth-instar larvae of the susceptible (SS) and abamectin-resistant (AB-Fd & AB-L) strains of CPB

| Post-treatment Interval (hr) | SS | AB-Fd | AB-L |
|---|---|---|---|
| | (% of total applied dose ± SD) | | |
| External Rinse | | | |
| 0 | 90.2 ± 5.0 | 93.2 ± 3.2 | 94.7 ± 3.0 |
| 1 | 40.6 ± 6.7 | 49.8 ± 10.9 | 49.2 ± 4.3 |
| 2 | 34.3 ± 4.7 | 33.7 ± 2.8 | 32.8 ± 2.1 |
| 6 | 15.8 ± 2.3 | 17.2 ± 3.8 | 22.2 ± 3.2[a] |
| Internal Extract | | | |
| 0 | 2.0 ± 1.8 | 1.2 ± 0.9 | 1.2 ± 1.0 |
| 1 | 21.8 ± 5.2 | 19.1 ± 7.3 | 25.2 ± 7.0 |
| 2 | 26.1 ± 4.6 | 22.9 ± 8.2 | 21.8 ± 1.8 |
| 6 | 37.6 ± 4.7 | 23.6 ± 5.8[a] | 25.3 ± 1.7[a] |
| Excrement Extract | | | |
| 0 | - | - | - |
| 1 | 18.3 ± 11.0 | 19.3 ± 3.8 | 22.4 ± 4.2 |
| 2 | 30.3 ± 12.2 | 36.4 ± 8.6 | 26.2 ± 0.9 |
| 6 | 27.8 ± 2.0 | 42.2 ± 11.7[a] | 35.3 ± 5.1[a] |

[a] Significantly different from the SS strain, *t* test, $P < 0.05$, N=4.

In Molecular Mechanisms of Insecticide Resistance; Mullin, C., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1992.

could be caused by a higher rate of excretion of the parent compound, to an increase in the level of water-soluble metabolites which are then more rapidly excreted or to a combination of both.

### **Differential** In Vitro **Activities using General Metabolic Substrates.**

Cytochrome P450 levels were significantly elevated (i.e., 60-90%) in the two abamectin-resistant strains compared to the SS strain (bottom, Table IV). However, oxidative activities (i.e., oxidases) were not increased for any of the general oxidative substrates tested and there was no concurrent increase in cytochrome c reductase activity or cytochrome $b_5$ levels (Table IV).

General esterase and carboxylesterase activities in the abamectin-resistant strains were also significantly enhanced compared to the SS strain (Table IV). This was most apparent in the carboxylesterase assay where there was over a 2-fold increase in carboxylesterase activity to $\alpha$-naphthyl butyrate in both abamectin-resistant strains compared to the susceptible strain. The overall increases in esterase activities indicates that either higher levels of esterases are being produced or esterases with enhanced activities (e.g., turn over rate, etc.) or substrate affinities have been selected.

A Lineweaver-Burk double reciprocal plot of carboxylesterase activity (Figure 5) indicates that there is no apparent change in substrate affinities between the susceptible and resistant strains since the $k_m$ is approximately equal (150 vs. 144 uM, respectively) but the $V_{max}$ is 2.5-fold higher (297 vs. 118 nmoles/min/mg protein) in the resistant strains. Using native polyacrylamide gel electrophoresis to separate the proteins of the 105,000g supernatant and determining carboxylesterase activity as above, it was possible to detect strain differences in their ability to hydrolyze $\alpha$-naphthyl butyrate by dianisidine staining (*20*). Total hydrolytic activity in the major staining band of the gel (i.e., largest peak identified by scanning desitometry) increased 1.3-fold in the AB-L strain compared to the SS strain, while carboxylesterase activity increased 2.6-fold (Figure 6). Interestingly, only 0.58 of the total hydrolytic activity of the SS strain was due to carboxylesterase activity, while this activity accounted for 0.86 of the total hydrolytic activity in the AB-L strain. These results further indicate that abamectin resistance may be in part attributable to increased levels of carboxylesterase(s).

There were no significant differences in glutathione-S-transferase activities between the susceptible and abamectin-resistant strains in their ability to metabolize CDNB or DCNB (Table IV).

**In Vivo Metabolism of [$^3$H]Avermectin B1a.** HPLC analysis with radiometric detection (personal communication, L. Crouch, 1990) of the excrement at 6 hr post-treatment revealed no significant difference of [$^3$H] avermectin B1a levels in any of the strains. However, significantly higher levels of 3" desmethyl avermectin B1a (3"desmethyl), 24-hydroxylmethyl avermectin B1a (24-OH), and an unidentified metabolite which eluted off the reverse-phase column at 14-15 min (i.e., Fraction 14) were associated with the abamectin-resistant strains compared to the SS strains (Table V). The AB-Fd strain had slightly elevated levels of all metabolites, which may explain why the AB-Fd had a slightly higher level of resistance to abamectin compared to the AB-L strain (Table I). The AB-L strain had significantly higher 24-OH and fraction 14 metabolite levels

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Table IV. *In vitro* metabolic activities of fourth-instar larvae of the susceptible (SS) and abamectin-resistant (AB-Fd & AB-L) strains of CPB

| Assays[a] | SS | AB-Fd | AB-L |
|---|---|---|---|
| | (nmole/min/mg protein ± SD) | | |
| Glutathione-S-Transferase (5)[b] | | | |
| CDNB | 129.4 ± 12.9 | 143.9 ± 10.5 | 140.2 ± 6.5 |
| DCNB | 8.0 ± 0.9 | 9.1 ± 0.9 | 8.6 ± 0.3 |
| General Esterases(4) | | | |
| α-Napthyl acetate | 356.5 ± 39.9 | 516.6 ± 11.5$^{c}$ | 505.7 ± 37.4$^{c}$ |
| α-Napthyl butyrate | 450.0 ± 72.3 | 689.4 ± 63.5$^{c}$ | 711.0 ± 0.3$^{c}$ |
| Carboxylesterases(4)[d] | | | |
| α-Napthyl acetate | 77.7 ± 6.7 | 97.6 ± 12.6$^{e}$ | 117.2 ± 8.1$^{c}$ |
| α-Napthyl butyrate | 73.3 ± 17.2 | 150.7 ± 43.8$^{c}$ | 193.7 ± 20.3$^{c}$ |
| Aliesterase (4) | | | |
| Methylthiobutyrate | 7.7 ± 0.7 | 7.2 ± 2.7 | 8.6 ± 1.9 |
| Oxidases (4) | | | |
| *O*-demethylation *p*-nitroanisole | 138.2 ± 45.0 | 158.8 ± 45.0 | 118.9 ± 42.5 |
| Methoxyresorufin | n.d.[f] | n.d. | n.d. |
| Microsomal ester cleavage | 124.5 ± 51.6 | 123.4 ± 26.9 | 118.0 ± 17.5 |
| Oxidative ester cleavage[g] | 25.6 ± 6.7 | 28.3 ± 9.2 | 20.9 ± 5.7 |
| Biphenyl hydroxylation | n.d. | n.d. | n.d. |
| NADPH-reductase cytochrome[c] | 36.9 ± 9.2 | 39.2 ± 13.6 | 43.3 ± 6.5 |
| | (pmole/mg protein ± SD) | | |
| Cytochromes (6) | | | |
| P420 | 139.2 ± 79.7 | 108.8 ± 32.3 | 116.4 ± 28.8 |
| P450 | 240.2 ± 79.4 | 457.5 ± 140.3$^{e}$ | 388.8 ± 113.9$^{e}$ |
| $b_5$ | 264.3 ± 35.1 | 221.2 ± 31.8 | 205.1 ± 63.4 |

[a] The following references detail the standard assay methods used: Glutathione-S-transferase (21); general esterases (22); carboxylesterases (23); aliesterase (24); O-demethylation (25, 26); microsomal and oxidative ester cleavage (27); biphenyl hydroxylation (28); NADPH-dependent cytochrome c reductase (29); cytochrome P450 and P420 (30); cytochrome $b_5$ (29).

[b] Number of replicates in parentheses (N).

[c] Significantly different from SS strain, *t* test, $P < 0.01$.

[d] Carboxylesterase activity measured by inhibiting acetylcholinesterase with eserine (0.1 mM) and arylesterases with PHMB (0.1 mM).

[e] Significantly different from SS strain, *t* test, $P < 0.05$.

[f] Not detected.

[g] Oxidative ester cleavage was measured by inhibiting membrane associated esterases activity to *p*-nitroacetate with DEF (0.1 mM).

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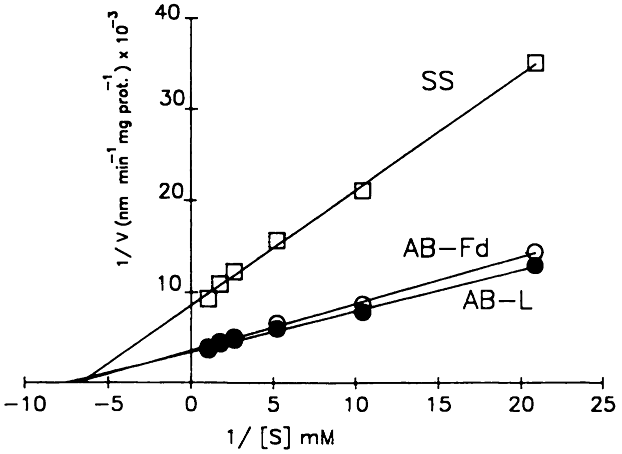

Figure 5. Lineweaver-Burk double reciprocal plot of carboxylesterase activity (α-napthyl butyrate hydrolysis in the presence of eserine and PHMB) in the susceptible (SS) and abamectin-resistant (AB-Fd & AB-L) strains of CPB.

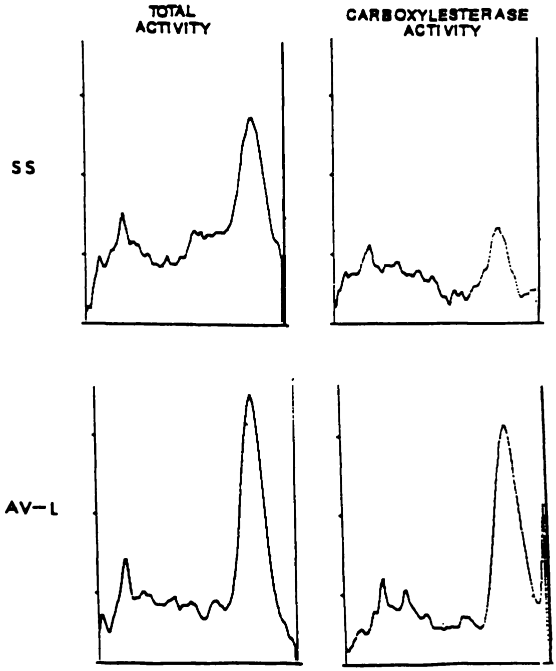

Figure 6. Scanning densitometry patterns of native polyacrylamide gel electrophoresis of total hydrolytic and carboxylesterase activities in the SS and AB-L strains of CPB.

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Table V. *In vivo* and *in vitro* metabolism of [$^3$H]Avermectin B1a by susceptible (SS) and abamectin-resistant (AB-Fd & AB-L) strains of CPB

| Assay and Metabolites | SS | AB-Fd | AB-L |
|---|---|---|---|
| | (% of applied dose in sample ± SD) | | |
| *In Vivo*[a] | | | |
| Avermectin B1a | 32.71 ± 3.61 | 35.80 ± 5.50 | 33.35 ± 4.45 |
| 3"Desmethyl | 1.26 ± 0.18 | 2.30 ± 0.28[b] | 1.51 ± 0.19 |
| 24-OH | 0.45 ± 0.01 | 1.16 ± 0.21[b] | 0.87 ± 0.21[c] |
| Fraction 14 | 0.58 ± 0.03 | 1.83 ± 0.69[c] | 1.50 ± 0.50[c] |
| *In Vitro* | | | |
| Microsomes (NADPH) | | | |
| Avermectin B1a | 77.00 ± 5.03 | 64.30 ± 11.07 | 72.19 ± 8.40 |
| 3"Desmethyl | 2.72 ± 0.21 | 6.37 ± 0.01[b] | 5.21 ± 0.72[b] |
| 24-OH | N.D.[d] | 1.50 ± 0.75[b] | 0.72 ± 0.27[b] |
| Fraction 14 | N.D. | 3.33 ± 1.00[b] | 1.34 ± 0.74[b] |

[a] Extract from excrement collected from CPB at 6 hr, N=3. [$^3$H]Avermectin B1a was applied at 0.46ng/larva.
[b] Significantly different from the SS strain, *t* test, $P < 0.01$, N=3.
[c] Significantly different from the SS strain, *t* test, $P < 0.05$, N=3.
[d] Not detected.

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but did not have a significantly higher level of 3"desmethyl avermectin B1a. The increased levels of water-soluble, oxidative metabolites strongly indicate this as a resistance mechanism in the AB-Fd and AB-L strains.

Interestingly, the major metabolite formed in all strains was 3"desmethyl avermectin B1a and is similar to the findings in rat (*31, 32*). However, unlike rat, the novel metabolite, fraction 14, was detected at the same or greater levels as 24-OH regardless of the strain but was particularly evident in the abamectin-resistant strains (Table V). Apparently, fraction 14 has a water solubility intermediary between 24-OH and 3"desmethyl, since these two metabolites eluted off the reversed-phase HPLC column at 6 and 19 min, respectively.

In Vitro **Metabolism of [$^3$H]Avermectin B1a.** The *in vitro* metabolism of [$^3$H]avermectin B1a produced similar results as in the *in vivo* studies (Table V). The 3"desmethyl metabolite formation was elevated 2.3-fold and 1.0-fold in the AB-Fd and AB-L strains, respectively. The fraction 14 and 24-OH metabolites were not detectable in the SS strain, while both abamectin-resistant strains had detectable levels of these metabolites. These metabolites are apparently formed by monooxygenases, since PBO-treated microsomes produced no metabolites in any strain, including fraction 14 (unpublished data, J. A. Argentine).

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## Conclusions

EMS treatment and abamectin selection resulted in two abamectin-resistant strains of CPB, AB-L and AB-Fd, respectively. Resistance levels appeared to stabilize at 23- and 15-fold for AB-Fd and AB-L strains, respectively. No cross-resistance to dieldrin was evident in either resistant strain. However, this does not rule out the possibility of a site-insensitivity resistance mechanism for abamectin, since dieldrin binds to the picrotoxinin site on the GABA-chloride channel. Abamectin resistance was determined to be autosomal, incompletely recessive, and polyfactorial for both strains.

PBO strongly synergized the toxicity of abamectin, particularly in the resistant strains. The use of PBO may lengthen the effective life span of abamectin, and if used before resistance develops, may possibly prevent this particular resistance factor from developing in the population.

Based on synergism, *in vivo* and *in vitro* metabolism and cytochrome P450 data, abamectin resistance is at least partly due to oxidative metabolism. This is not surprising since abamectin metabolism in mammals is principally oxidative (*31, 32*). In all strains, the major metabolite detected was 3"desmethyl avermectin B1a. The level of this oxidative metabolite was significantly elevated in both abamectin-resistant strains under both *in vivo* and *in vitro* assay conditions. Interestingly, a new but unidentified metabolite (fraction 14) was found and its formation was enhanced principally in the abamectin-resistant strains.

Evidence suggests carboxylesterases may also be involved in abamectin resistance. Synergism to DEF was not as high as with PBO, but was higher in the abamectin-resistant strains compared to SS strain. Carboxylesterase activity as judged by α-naphthyl butyrate hydrolysis was much higher in the abamectin-resistant strains than the SS strain and this difference was due to an elevated Vmax rather than to a change in affinity (Km) of the enzyme. Because no radiolabeled hydrolysis products of abamectin were observed, it is unclear if hydrolytic degradation of abamectin is a resistance mechanism. Apparently, the extraction conditions used to isolate abamectin metabolites can result in the reestablishment of the cyclic lactone moiety of abamectin. This makes a clear identification of this hydrolytic product impossible at this time (personal communication, L. Crouch, 1990). Thus, the increase in carboxylesterases could be incidental. Alternatively, carboxylesterases may be acting as a sequestering agent against abamectin. A carboxylesterase in *Myzus persicae* has been shown to act as a sequestering agent against a variety of insecticides (*33*). Preliminary evidence from this laboratory indicates that abamectin is a competitive inhibitor of carboxylesterase activity but has a very low affinity for the enzyme(s) (unpublished data, H. Lin).

Both oxidative and hydrolytic metabolism associated with abamectin resistance in CPB appears to be due to increased levels of enzyme or enhanced activity rather than a change in substrate specificity. An increase in enzyme levels could be due to two separate mutations involved in the regulation of each enzyme, or one major regulatory gene may be involved. Gene amplification, which has occurred in esterase genes involved in insecticide resistance, could also be responsible for the increase levels of enzyme. This obviously will be an interesting area of study in the future.

Finally, it appears that both AB-Fd and AB-L strains have the same or very similar mechanisms of resistance to abamectin. This demonstrates the merit and possible utility of using EMS as a means to generate resistance in insects prior to the commercial use of an insecticide.

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## Chapter 21

# Detoxification of Mycotoxins by Insects

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Resistance to aflatoxin $B_1$ in *Drosophila melanogaster* is conferred by at least two chromosomes and cytoplasmic factors, and it is thought that unspecific monooxygenases may be involved. Resistance to $\alpha$-amanitin in *D. melanogaster* is due to an altered target site, RNA polymerase II. Relative resistance to aflatoxin $B_1$ and griseofulvin in *Spodoptera frugiperda* is due to lower rates of activation and higher rates of detoxification compared to *Helicoverpa zea*. Fungus-feeding larvae of *Carpophilus hemipterus* are able to hydrolyze a model trichothecene substrate at about 10-fold the rate of *H. zea* and *S. frugiperda*.

Mycotoxins are secondary metabolites produced by fungi that are toxic to animals, including humans. They represent just a small portion of the many secondary metabolites produced by fungi (*1,2*). Most mycotoxins are produced by molds in the genera *Aspergillus, Penicillium,* and *Fusarium*. Some representatives and their effects are listed in Table I, and corresponding structures are shown in Figure 1.

Unlike plant secondary metabolites, the recognition that fungal secondary metabolites such as mycotoxins can act as defensive substances has occurred only recently (*3*). Nevertheless, the effects of several mycotoxins on insects have been studied to some degree (see reviews *4,5*). Studies on insects have been prompted by desires to examine the defensive capabilities of mycotoxins, to test insects as alternative bioassay indicators, and to search for new insecticides or novel bioactive metabolites that provide leads for new insecticides.

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ACS Symposium Series; American Chemical Society: Washington, DC, 1992.

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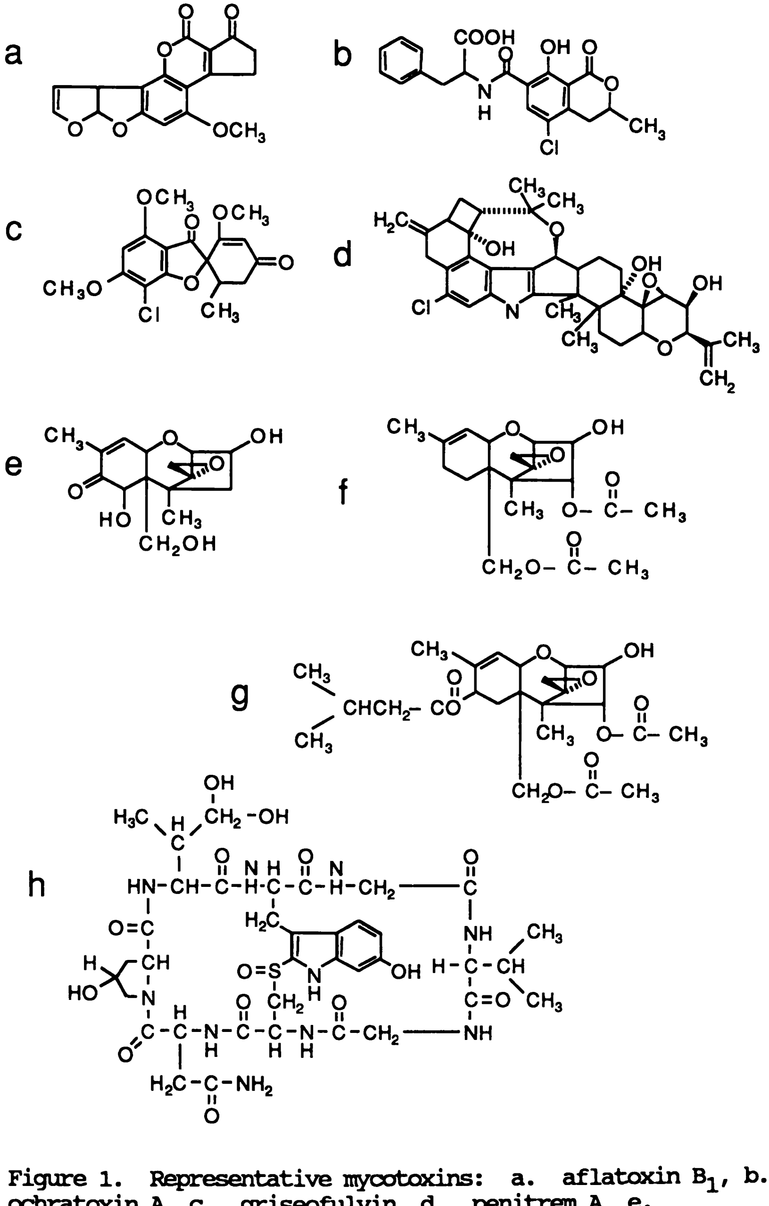

Figure 1. Representative mycotoxins: a. aflatoxin $B_1$, b. ochratoxin A, c. griseofulvin, d. penitrem A, e. deoxynivalenol, f. diacetoxy-scirpenol, g. T-2 toxin, h. α-amanitin.

Table I. Representative Mycotoxins and Their Effects

| | |
|---|---|
| *Aspergillus* | |
| aflatoxins | hepatotoxic, carcinogenic |
| sterigmatocystin | carcinogenic |
| ochratoxin A | nephrotoxic, carcinogenic |
| *Penicillium* | |
| citrinin | hepatotoxic |
| griseofulvin | carcinogenic, teratogenic |
| patulin | toxic, carcinogenic |
| rubratoxins | hepatotoxic |
| penitrems | tremorgenic |
| penicillic acid | carcinogenic |
| *Fusarium* | |
| T-2 toxin | dermal necrosis, hemorrhagic |
| deoxynivalenol | emetic, nephrotoxic |
| diacetoxyscirpenol | dermal necrosis, hemorrhagic |
| zearalenone | estrogenic |

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## Mycotoxin Effects on Insects

For the most part, carcinogenicity is not of concern in insect studies due to the rarity of insect cancers. Thus, the reported effects of mycotoxins on insects include acute toxicity, reduction in growth rates, morphological, histological and reproductive changes. The two most extensively studied groups of mycotoxins are the aflatoxins and the trichothecenes. These compounds presumably act on insects in a manner similar to the way they act on mammals, by binding with DNA (aflatoxins) or by inhibiting protein synthesis (both). Insects fed these compounds may die, have reduced growth rates, or reduced fecundity. Aflatoxin $B_1$ is reported to induce recessive lethal mutants in *Drosophila melanogaster* (*6*). The tremorgenic mycotoxins interact with the insect nervous system presumably as they do in vertebrates, by affecting $\gamma$-aminobutyric acid (GABA), glutamic, or other tramsmitters/receptor systems (*5*). Those that affect nitrogen regulation and manifest their effects on the mammalian kidney can also affect the corresponding structures in insects, the Malpighian tubules (*7*). In fact, virtually all mycotoxins have some effect on insects at naturally occurring concentrations (typically 25 ppm) (*5*). Thus, it is easy to understand why mycotoxins are considered as defenses against insects, as well as mammals. The implications of this concept are that all of the reactions and interrelationships known for insects and plant secondary metabolites can also be applied to insects and mycotoxins.

**Insect "Resistance" to Mycotoxins**

Insect resistance to mycotoxins may have evolved through continuous exposure and/or targeted feeding. Resistance to plant secondary metabolites and synthetic insecticides is sometimes thought to result from predisposed adaptations to (for example) plant secondary metabolites that have particular functional groups in common. The same situation is relevant for mycotoxins. For example, detoxifying enzyme systems that evolved in insects to dealkylate plant allelochemicals may be capable of dealkylating mycotoxins. Possibly, insect resistance to mycotoxins is ancestral to resistance to plant allelochemicals. Based on speculated times of origin, insects (*8*) and fungi (*9*) have been interacting for nearly 400 million years. This is approximately 275 million years longer than the period that insects have been interacting with flowering plants (based on a time of origin 125 million years ago - *10*). Parallel evolutionary development of resistance to mycotoxins and plant allelochemicals by insects is also a possibility. Interestingly, one of the "oldest" insect groups, the cockroaches, is also one of the most resistant to aflatoxins (*11*). The aflatoxins themselves may represent the present day biosynthetic endpoint for a progression of precursors that show a decreasing toxicity to insects the further they are removed from aflatoxins (*12*).

Insects with similar host ranges have different sensitivities to mycotoxins even though they would not be expected to have adapted to mycotoxins due to targeted feeding. One example is *Spodoptera frugiperda* and *Helicoverpa zea*, both of which may feed on corn or other plants occasionally contaminated with mycotoxins. Based on sublethal effects at 0.25 ppm (*13*), *H. zea* is about 10-fold more sensitive to aflatoxin $B_1$ than *S. frugiperda*. In contrast, *S. frugiperda* is more sensitive than *H. zea* to the tremorgen roseotoxin B at 25 ppm in diets (100% vs. 38% mortality, respectively). The converse is true for the tremorgen penitrem A (20% vs. 80% reduction in growth rates, respectively, at 0.25 ppm in diets) (*14*). Griseofulvin, a mycotoxin that is also used pharmaceutically to treat fungal skin infections, is more toxic to *H. zea* than *S. frugiperda* (Dowd, P.F., unpublished data - see following discussion). On the other hand, most trichothecenes are of similar toxicity to both insects (*5,15-17*).

There is also variability in susceptibility of different strains of *Drosophila melanogaster* to aflatoxin $B_1$. At 400 ppb, all of the Crimea strain died, 24% of the Swedish-C strain reached adulthood and approximately 70% of the Hikone-R, Lausanne-S, and Oregon-R strains reached adulthood (*18*). These differences in susceptibility suggest that some sort of resistance mechanism(s) are present.

There are other examples where continuous exposure has apparently produced resistance to mycotoxins in insects. Stored product beetles appear relatively resistant to ochratoxin A and T-2 toxin (*19*) compared to stored product caterpillars (*20*). Possibly the beetles are less mobile than the moths and have experienced greater selection pressure when stored materials have become contaminated with mycotoxin-producing fungi.

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There are also cases where targeted feeding on mycotoxin-contaminated materials has produced high levels of mycotoxin resistance. The different species of *Drosophila* that feed on mushrooms, potentially containing the toxin $\alpha$-amanitin, are much less susceptible to $\alpha$-amanitin than are unadapted, fruit-feeding species Fruit-feeding *Drosophila* species *D. melanogaster, D. pseudoobscura* and *D. immigrans* were adversely affected by dietary concentrations of $\alpha$-amanitin at 50 ppm or less, while the mushroom feeding *D. recens, D. putrida* and *D. tripunctata* were not affected (*21*). Similarly, the mushroom-feeding *D. recens, D. falleni* and *D. phalerata* were able to develop at least to pupae at $\alpha$-amanitin concentrations of 50 ppm while the closely related but detritous-feeding *D. quinaria, D. palustris*, and *D. subpalustris* were not (*22*). However, one strain of *D. melanogaster* was also resistant to $\alpha$-amanitin (*21*). The ability to feed on amanitin-containing mushrooms also appears to rid the flies of parasitic nematodes, which are adversely affected by the $\alpha$-amanitin (*22*).

Large sclerotia (long term survival structures of fungi) of the ergot fungus *Claviceps* spp. contain a number of neuroactive compounds, and are fed on by phalacrid beetles (Coleoptera: Phalacridae) (*23,24*). Finally, sap beetles (Coleoptera: Nitidulidae) are reported to feed on and spread a variety of mycotoxigenic fungi (*25*). Both adults and larvae of a representative sap beetle, *Carpophilus hemipterus* are only slightly, if at all, affected by short term exposures to representative *Aspergillus, Penicillium* and *Fusarium* mycotoxins at 25 ppm, including aflatoxin $B_1$, sterigmatocystin, citrinin, cyclopiazonic acid, penicillic acid, diacetoxyscirpenol, deoxynivalenol, and T-2 toxin (*5*). Thus, there are a number of insects that are relatively resistant to mycotoxins. In this review, the resistance mechanisms present in *D. melanogaster, S. frugiperda, H. zea* and *C. hemipterus* will be emphasized.

## Resistance Mechanisms

Although not specifically adapted for feeding on mycotoxin-containing materials, the strains of *D. melanogaster* resistant to aflatoxin $B_1$ and $\alpha$-amanitin have been investigated in some detail. Unfortunately, initial attempts at increasing resistance levels in the aflatoxin-resistant *D. melanogaster*, by selection for 18-20 generations, yielded strains with less than a 3-fold increase (*26*). Isogenic lines of Oregon-R and Lausanne-S were approximately 3 x more susceptible to 1.0 ppm of dietary aflatoxin $B_1$ than nonisogenic ones (*27*). This information suggests that the strains have not attained "true resistance" whereby genes conferring resistance have assorted appropriately. Hybrids between resistant (Lausanne-S) and susceptible (Florida-9, Canton-S, Swedish-C) strains were intermediate in resistance, with 85% of the Lausanne-S, 30% of the hybrids, and none of the susceptible strains surviving 1050 ppb of aflatoxin $B_1$ in diets (*28*). The authors suggested that multiple genes were likely to be involved (*28*).

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Chromosomal substitution studies initially indicated that autosomal genes on chromosomes 2 and 3 in the Lausanne-S strain control the resistance to aflatoxin $B_1$ (*29*). However, diallele analysis indicated that nuclear gene differences (chromosome X and 2) were responsible for differences in resistance, that some of these genes showed additive interactions, and that some of the gene expression (on chromosome 2) is regulated by cytoplasmic factors (*30*). As a result of these observations, the authors suggested that while quantitative/qualitative differences in P-450 xenobiotic-metabolizing enzymes (unspecific monooxygenases) may be partly involved, this did not explain the cytoplasmic-nuclear gene interactions (*30*). Unfortunately, the mechanisms of resistance to aflatoxin $B_1$ in these strains have not been determined.

The mechanism(s) for resistance to α-amanitin for the C-4 strain of *D. melanogaster* has been determined, and involves an altered target site. The α-amanitin appears to cause toxic effects by binding to RNA-polymerase and thereby inhibiting its function and subsequent protein synthesis. Jaenike et al. (*21*) found that compared to other *Drosophila* spp., including those resistant to α-amanitin, the RNA-polymerase II of the resistant strain of *D. melanogaster* retained at least 50% of its activity at concentrations of 5 ppm of α-amanitin *in vitro*, while that of the other *Drosophila* spp. was inhibited greater than 50% by α-amanitin at 0.1 ppm. Mechanisms of α-amanitin resistance in the mushroom-feeding species of *Drosophila* have not been determined.

Research conducted in our laboratory has concentrated on *S. frugiperda* and *H. zea* as representative insects that have differing susceptibilities to mycotoxins, and yet are not specifically adapted to feeding on mycotoxin-contaminated materials. As indicated before, these species have obvious differences in susceptibility to some, but not all, mycotoxins. In addition, they have been well studied for responses to, and metabolism of, plant allelochemicals and insecticides. A few "case studies" will now be discussed to indicate potential resistance mechanisms to mycotoxins present in unadapted insects, which might be considered cases of "predisposition". Although these studies are oriented towards activities of detoxifying enzymes, it is just as likely that other detoxifying mechanisms, such as sequestration or excretion, could also be involved.

When NADPH-associated oxidation assays were performed using midguts from *H. zea* and *S. frugiperda*, only two of over a dozen mycotoxins tested were significantly metabolized (Dowd, P.F., unpublished data). One of these, zearalenone, is a steroid-based compound that may be metabolized by enzymes associated with the regulation of ecdysone or other steroids in insects. The other, sterigmatocystin, is a biosynthetic precursor for aflatoxin $B_1$, and has moities (e.g. double bonds, alkyl groups) that also may be activated or detoxified.

Aflatoxin $B_1$ is an important *Aspergillus* mycotoxin and was selected for more detailed studies. In vertebrates and microorganisms, aflatoxin $B_1$ can undergo activating (generation of a reactive epoxide at the 2,3 position) and detoxifying (*O*-demethylation, glutathione conjugation) reactions. As indicated earlier, *S. frugiperda* is less sensitive to aflatoxin

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$B_1$ than *H. zea*. Thus, it was of interest to investigate potential detoxifying strategies to determine the cause of "resistance" of aflatoxin $B_1$ in *S. frugiperda* compared to *H. zea*.

To facilitate this investigation, a reverse-phase TLC method was developed. This method readily separated bound material (origin), the parent aflatoxin $B_1$ (Rf ca. 0.5), potentially activated compounds (lower Rf than aflatoxin $B_1$) and detoxified metabolites, with the more polar (and presumably less toxic) metabolites having a higher Rf than aflatoxin $B_1$. Different cofactors, such as glutathione and NADPH, were used to determine potential types of enzymes involved. The intense and characteristic blue fluorescence of aflatoxin $B_1$ and some of the other metabolites at the concentration used provided an immediate indication of metabolic strategies. Based on relative polarities and the limited number of standards available, *H. zea* is more likely to activate, and less likely to produce relatively polar, less toxic metabolites than is *S. frugiperda* (Figure 2). Quantitation and identification of the metabolites is still in progress. Although NADPH enhanced production of some metabolites in *H. zea*, it is presently unknown whether the glutathione conjugates are formed from the activated, intermediate epoxide, as occurs in rats (*31*). Interestingly, the fungus can compensate for a predator's oxidative detoxification of aflatoxin $B_1$ by producing large quantities of kojic acid, which inhibits oxidative detoxification of other compounds (*13,15*). Whether kojic acid is selective for detoxifying vs. activating monooxygenases is presently unknown.

A representative *Penicillium* mycotoxin with differential toxicity to *S. frugiperda* and *H. zea* is griseofulvin. Again, different rates of detoxification and activation appear to explain the disparity in toxicity in these two insect species. However, photoactivation of this compound (Dowd, P.F., unpublished data) is a further complicating factor. Griseofulvin was metabolized rapidly in midguts of *H. zea*, and the metabolism was almost completely inhibited by piperonyl butoxide (Table II). In *S. frugiperda*, metabolism was about half the rate of that in *H. zea*, and piperonyl butoxide only inhibited metabolism by about 50%. However, in oral toxicity assays, piperonyl butoxide was more effective in synergizing the toxicity of griseofulvin in *S. frugiperda* than *H. zea* (where toxicity was antagonized). Although a number of explanations are possible, apparently in *H. zea* some sort of monooxygenase-dependent activation occurs, while in *S. frugiperda* monooxygenase-dependent detoxification is more important. These metabolism profiles are consistent with the differences in toxicity.

As discussed earlier, the toxicity profiles for most trichothecenes are very similar for *H. zea* and *S. frugiperda*. Detoxifying enzymes can often be induced by plant allelochemicals or insecticides, which is presumably due to *de novo* protein synthesis (*32*). As foreign compounds, trichothecenes may potentially induce detoxifying enzymes in insects, but as protein synthesis inhibitors, trichothecenes could also depress these same activities. Interestingly, when *H. zea* and *S. frugiperda* were used to examine this relationship,

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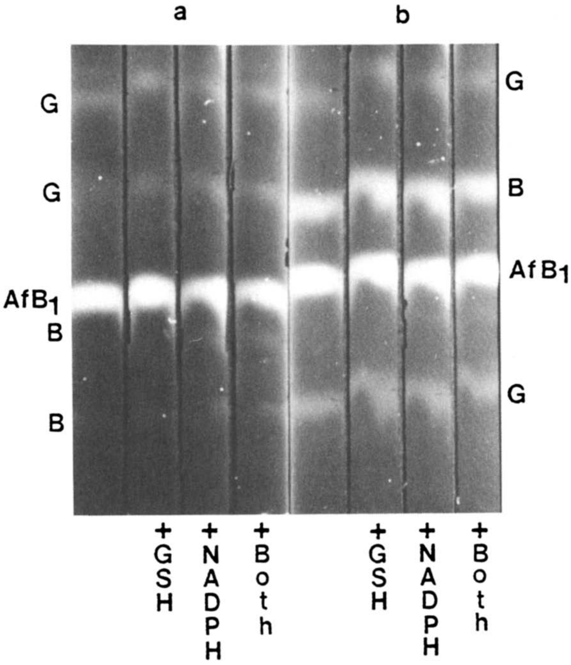

Figure 2. Aflatoxin $B_1$ and metabolites separated by thin-layer chromatography and visualized under long wave UV light. a. *H. zea*, b. *S. frugiperda*. Bluish spots indicated with "B" and greenish spots indicated with "G".

unspecific monooxygenase and glutathione transferase activities were sometimes induced by 250 or 25 ppm of deoxynivalenol, T-2 toxin, and diacetoxyscirpenol (*16*). Surprisingly (based on earlier work with esterases, which indicates levels of esterase induction are typically very low, e.g. *32*), a new esterase (as indicated by gel electrophoresis and stain for 1-naphthyl acetate esterase) was also induced in both insects by these concentrations of trichothecenes (*16*). Based on indirect assays, such as using 1-naphthyl acetate as a competitive inhibitor, this esterase appeared to be responsible for hydrolyzing acetylated trichothecenes, including the model substrate monoacetoxyscirpenol (*16*). Thus, detoxifying enzyme systems in insects can also respond to mycotoxins by synthesizing greater levels of detoxifying enzymes. Although not yet examined, this capability may also be involved in insect resistance to mycotoxins.

Table II. Toxicity and Metabolism of Griseofulvin Alone and in Combination with Piperonyl Butoxide

| | *Oral toxicity* (% Control weight) *- piperonyl butoxide* | *+ piperonyl butoxide* |
|---|---|---|
| *S. frugiperda* | | |
| - griseofulvin | 100.0 | 79.7 |
| + griseofulvin | 82.0 | 43.5 |
| *H. zea* | | |
| - griseofulvin | 100.0 | 74.9 |
| + griseofulvin | 45.0 | 34.3 |

| | *Metabolism* (%) *- piperonyl butoxide* | *+ piperonyl butoxide* |
|---|---|---|
| *S. frugiperda* | 30.4 | 15.2 |
| *H. zea* | 60.8 | 3.7 |

Metabolism is based on 3 hour incubations of 1 intact gut with 100 nmole of griseofulvin.

## Insects Resistant to Mycotoxins

In spite of the number of examples given earlier where selected species of insects have demonstrated resistance to mycotoxins, relatively few studies have investigated the resistance mechanisms in insects that frequently or preferentially feed on materials contaminated with mycotoxins.

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As mentioned earlier, the resistance mechanism of the mushroom-feeding *Drosophila* spp. to α-amanitin has not been determined. The resistance mechanism of the phalacrid beetles that feed on ergot is also unknown. However, symbionts in the cigarette beetle, *Lasioderma serricorne* (Coleoptera: Anobiidae), may contribute to this insect's resistance to mycotoxins. The apparent detoxification of ochratoxin A by symbionts has been detected histochemically (*33*). Cultures of the symbiont can apparently utilize mycotoxins such as ochratoxin A, deoxynivalenol, citrinin, mycophenolic acid, and sterigmatocystin as carbon sources, also suggesting an ability to detoxify them (*34*). Studies on the ability of cultured symbionts to detoxify aflatoxin $B_1$ are in progress. However, metabolic studies in the presence and absence of symbionts in insects fed mycotoxins are needed to clarify this possibility.

Sap beetles also are resistant to the effects of mycotoxins. The model trichothecene monoacetoxyscirpenol was primarily detoxified by hydrolysis at 8 to 10 fold the rate of *H. zea* and *S. frugiperda* (*35*). This information suggests that enhanced enzymatic detoxification contributes to trichothecene resistance in sap beetles. Studies with aflatoxin $B_1$ (Dowd, P.F., unpublished data) indicated NADPH-enhanced production of a metabolite. This metabolite had a slightly lower Rf than aflatoxin $B_1$ by reversed-phase TLC, but quantitation is not yet complete. Possibly, altered target sites or simple excretion or sequestration are also involved in resistance.

## Summary and Utility

The preceding discussion has indicated that, as is the case for insect resistance to plant allelochemicals, enzymatic detoxification contributes to mycotoxin resistance in insects, although altered target sites are also a possibility. Similar to the case for plant allelochemicals and insecticides, insect systems respond to dietary mycotoxins by synthesizing new or increased levels of detoxifying enzymes. Resistance to mycotoxins has allowed some insects to exploit substrates that are unavailable to other species. In some cases, insects resistant to mycotoxins may deliberately carry mycotoxin-producing fungi and inoculate materials so that the subsequent production of mycotoxins excludes competing insects (*25*).

Many questions remain to be answered, because there is only a limited amount of research that has been performed on mycotoxin resistance in insects. Mycotoxins (*4,14*), other fungal metabolites (*5*) or their derivatives are potentially a good source for novel insect control agents. By studying resistance mechanisms to fungal metabolites such as mycotoxins, it may be possible to evaluate the potential for resistance development as compared to plant-derived or other microbially-produced insecticides. Information gained from these studies may permit determination of whether mycotoxin resistance mechanisms are ancestral to others and help direct the design of new insecticides.

The contamination of foodstuffs and feedstuffs by mycotoxins continues to be a worldwide problem. Insects are probably the only group of organisms that have widespread resistance to mycotoxins.

Isolation and characterization of receptors and enzymes involved in detoxification may reveal mechanisms that can be applied to other situations. For example, incorporation of insect-derived genes for mycotoxin detoxification into appropriate microorganisms may allow for bioremediation of toxin-contaminated animal feeds or chemical feedstocks. Even the insects could be used as bioconversion mechanisms. In conclusion, the study of insect resistance mechanisms to mycotoxins may yield information applicable to solutions for a variety of problems associated with both insecticide resistance and mycotoxin contamination.

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## Chapter 22

# Interactions of *N*-Alkylamides with Voltage-Sensitive Sodium Channels

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Radioligand binding and sodium flux assays were employed to define the actions of a series of *N*-alkylamides on sodium channels. In the presence of a saturating concentration of scorpion (*Leiurus quinquestriatus*) venom, seven insecticidal compounds were active as both inhibitors of [$^3$H]batrachotoxinin A 20α–benzoate ([$^3$H]BTX-B) binding and stimulators of sodium uptake. Three non-insecticidal compounds in this series were also active as inhibitors of [$^3$H]BTX-B binding but failed to stimulate sodium uptake. In this chapter, we summarize data from biochemical studies which suggest that the insecticidal action of *N*-alkylamides results from their binding to site 2, the activator recognition site, of voltage-dependent sodium channels. These findings illustrate the significance of multiple binding domains of the sodium channel as target sites for insect control.

The *N*-alkylamides are a class of unsaturated, lipophilic compounds isolated from members of the plant families Compositae, Piperaceae and Rutaceae (*1*, *2*). Although the bioactivity of these chemicals was described over a century ago, little was known until recently regarding their mechanism of action.

Whereas low levels of insecticidal activity are reported for naturally-occurring *N*-alkylamides (*1-4*), recent efforts in a number of laboratories have resulted in the synthesis of analogues with enhanced toxicity to insects (*5-12*). The structure of pellitorine, an *N*-alkylamide first isolated from roots of *Anacyclus pyrethrum* (Compositae), has proven useful as a prototype for the synthesis of compounds (Figure 1) with stabilities and potencies that approach those necessary for practical application (*10*, *12*). Of particular interest are reports that the insecticidal activity of these analogues is maintained (*12*) or enhanced (*10*) in bioassays against insects with genotypes (e.g. *super-kdr*) conferring reduced neuronal sensitivity to pyrethroids.

Insecticidal *N*-alkylamides produce physiological effects that are qualitatively similar to those of the pyrethroids. In preliminary neurophysiological studies, these compounds induce repetitive activity followed by conduction block in housefly nerves (*13*). In addition, *N*-alkylamides were shown to suppress peak sodium current and induce slowly decaying tail currents in voltage-clamped locust nerve cell bodies in culture (*14*). However, unlike deltamethrin (DTM), *N*-alkylamides inhibit (rather

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than stimulate) the veratridine (VTD)-dependent release of acetylcholine from preloaded synaptosomes from the cockroach central nervous system (*15*). These results implicate the voltage-sensitive sodium channel as the principal site of action for these compounds.

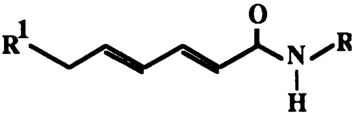
Whereas results from electrophysiological studies suggest an interaction between *N*-alkylamides and the sodium channel, the methods employed lack the resolution necessary to identify and characterize the binding site associated with insecticidal activity. Specific binding domains on sodium channels have been identified for a variety of neurotoxic agents (*16*) including the sodium channel blockers tetrodotoxin (TTX) and saxitoxin (site 1), the activators batrachotoxin (BTX) and veratridine (VTD) (site 2), polypeptide toxins from venoms of sea anemones and scorpions (sites 3 and 4) and the brevetoxins and ciguatoxins (site 5). In addition, less well-characterized sites have been proposed for pyrethroid insecticides and DDT analogues (*17-20*), Pumiliotoxin B (PTX-B) (*21*), and a polypeptide toxin isolated from *Goniopora* corals (*22*).

In this chapter, we review results from biochemical studies which suggest that insecticidal *N*-alkylamides act as partial agonists at site 2 of sodium channels from mouse brain preparations (*23, 24*) and, therefore, represent a new class of sodium channel activators. Also novel is the finding that these compounds exert their insecticidal effect by binding at a site different from that of the pyrethroids and DDT.

## Methods

**Chemicals.** The *N*-alkylamides used in this study (Table I) were generously provided by N. Janes (Rothamsted Experimental Station, Harpenden, England). In bioassays with house flies (*Musca domestica* L.) and mustard beetles (*Phaedon cochleariae* Fab.), the insecticidal potencies of BTG 502 and compounds **A-F** were 1-8% of that measured for bioresmethrin whereas **G-I** were non-toxic (*9, 25*, N. F. Janes, personal communication). PTX-B was a gift from J. Daly (National Institute of Arthritis, Metabolism and Digestive Disease, Bethesda MD). The sources of other chemicals used in these assays are published elsewhere (*23*).

Table I. Structures of *N*-Alkylamides Tested



| Designation | R1 | R2 |
|---|---|---|
| BTG 502 | 5-bromonaphth-2-yl- | $-CH(CH_3)CH(CH_3)_2$ |
| **A** | $C_6H_5$- | $-CH_2CH(CH_3)_2$ |
| **B** | $C_6H_5$- | $-CH(CH_3)CH(CH_3)_2$ |
| **C** | 3,5-difluorophenyl- | $-CH_2C(CH_3)_3$ |
| **D** | dibenzofuran-3-yl- | $-CH_2C(CH_3)_3$ |
| **E** | $CH_3(CH_2)_5$- | $-CH_2CH(CH_3)_2$ |
| **F** | $C_6H_5(CH_2)_6$- | $-CH_2CH(CH_3)_2$ |
| **G** | $C_6H_5CH_2$- | $-CH_2CH(CH_3)_2$ |
| **H** | (3*E*,5*E*)-*N*-(1,2-dimethyl)-propyl-6-phenylhexa-3,5-dienamide | |
| **I** | (2*E*,4*E*)-*N*-(2-methylpropyl)-5-phenylpenta-2,4-dienamide | |

Source: Adapted from ref. 24.

**Preparation of Synaptoneurosomes.** Synaptoneurosomes were prepared from the brains of male ICR mice (Blue Spruce Farms, Altamont, NY) using the method of Brown (*26*) with slight modifications (*23*).

**BTX-B Binding Assays.** Inhibition of the specific binding of [$^3$H]batrachotoxinin A 20α–benzoate ([$^3$H]BTX-B) was measured by the method of Catterall et al. (*28*) as modified by Ottea et al. (*23*). Specific binding was calculated as the amount of total binding displaced by 500 μM VTD. Data presented are means from three or four triplicate assays using freshly prepared synaptoneurosomes for each assay.

**Sodium Uptake Assays.** The uptake of $^{22}$Na$^+$ into synaptoneurosomes was measured by the method of Tamkun and Catterall (*27*) as modified by Bloomquist and Soderlund (*19*). Methods for these assasys are described in detail elsewhere (*23*). Unless otherwise specified, values reported here are corrected for uptake measured in the presence of scorpion (*Leiurus quinquestriatus*) venom (ScV) alone. Data points represent results of three to six triplicate experiments using freshly prepared synaptoneurosomes for each assay.

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## Results and Discussion

**Effects of *N*-Alkylamides on [$^3$H]BTX-B Binding.** All of the *N*-alkylamides tested inhibited the specific binding of [$^3$H]BTX-B. BTG 502 was the most potent inhibitor producing half-maximal levels of inhibition at 1.43 μM (Table II). Scatchard analysis of equilibrium binding data revealed that BTG 502 produced a concentration-dependent decrease in the affinity of sodium channels for [$^3$H]BTX-B but had no significant effect on binding capacity (Figure 2). Equilibrium dissociation constants calculated for incubations with BTG 502 concentrations of 0, 3 and 10 μM were 50, 101 and 235 nM, respectively. Kinetic studies were performed to ascertain whether this decrease in binding affinity resulted from a BTG 502-dependent alteration in the rate of formation or dissociation of the site 2/BTX-B complex. Following equilibration of membranes with [$^3$H]BTX-B, the addition of BTG 502 (3 μM) had no significant effect on the subsequent rate of dissociation. However, in association experiments, BTG 502 (3μM) reduced the initial rate of formation of the receptor-ligand complex by nearly two-fold (data not shown).

Both toxic and non-toxic *N*-alkylamides inhibited the specific binding of [$^3$H]BTX-B (Figure 3). In preliminary tests, **A**-**I** (60 μM) were incubated in the presence of a saturating concentration of ScV (30 μg). Under these conditions, inhibition produced by **C** was nearly complete (91.6%) whereas levels of inhibition in the presence of the other insecticidal analogues were within the range of 37.5% (**F**) to 76.3% (**D**). In assays with the non-insecticidal analogues, inhibition by **G** (75.2%) was comparable to that measured with the insecticidal analogues **D** and **E**, while **H** and **I** were relatively poor inhibitors.

In dose-response experiments, all of the compounds produced incomplete levels of inhibition of the specific binding of [$^3$H]BTX-B at the highest concentration tested (75 μM; cf. **C** and **A** in Figures 4 and 5, respectively). The dibenzofuranyl-substituted analogue, **D**, was a potent inhibitor of binding with a $K_i$ of 9.22 μM (Table II) and produced maximal levels of inhibition (76.4%) at 30 μM (data not shown). Of the insecticidal compounds, **F** was the least effective inhibitor of [$^3$H]BTX-B binding with an estimated $K_i$ of 244 μM. The mean $K_i$ value measured in assays with the non-toxic compound, **G** (15.5 μM), was similar to that measured for the insecticidal analogues **C** and **E**.

Inhibition data were subjected to Hill analyses to ascertain further the nature of the effect of the *N*-alkylamides on [$^3$H]BTX-B binding. For all of the compounds tested (with the exception of **F**), the slopes of Hill plots for displacement of [$^3$H]BTX-B (Table II) were not significantly different from one (Student's t-test, $p < 0.05$)

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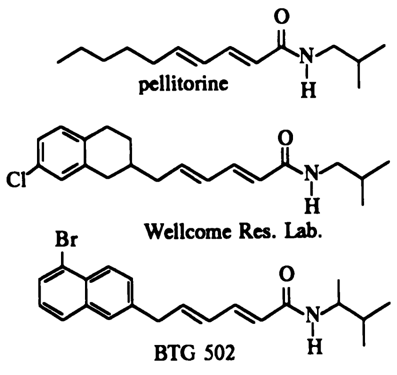

Figure 1. Natural (pellitorine) and synthetic *N*-alkylamides.

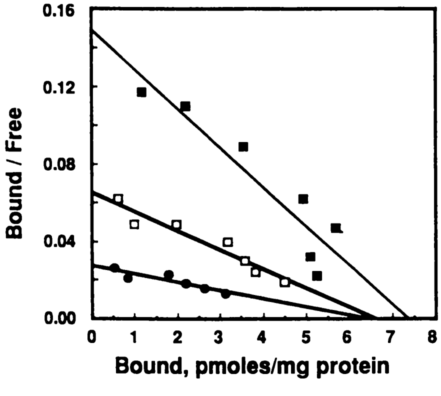

Figure 2. Scatchard analyses of the displacement of [$^3$H]BTX-B (10 nM) by increasing concentrations of BTX-B in the absence (closed squares) or presence of BTG 502 at concentrations of 3 μM (open squares) or 10 μM (circles). (Reproduced with permission from ref. 23. Copyright 1989 The American Society for Pharmacology and Experimental Therapeutics).

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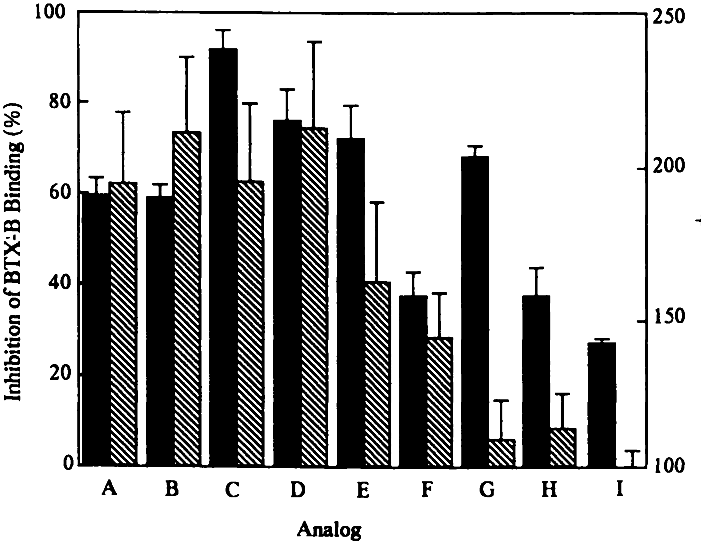

Figure 3. Inhibition of [$^3$H]BTX-B binding (solid bars) and enhancement of sodium uptake (hatched bars) by *N*-alkylamides. (Reproduced from ref. 24. Copyright 1990 American Chemical Society).

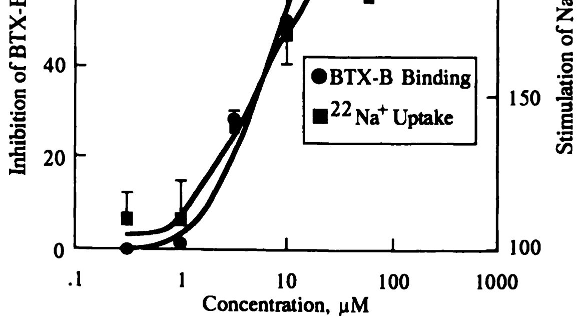

Figure 4. Concentration-dependent inhibition of [$^3$H]BTX-B binding (circles) and activation of sodium uptake (squares) by C. (Reproduced from ref. 24. Copyright 1990 American Chemical Society).

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suggesting that these compounds were acting as competitive inhibitors of binding at site 2. In contrast, the Hill slope estimated for F was 0.28. This finding is indicative of an effect of this compound at a sodium channel binding domain other than site 2. Such an interaction may involve either negative cooperativity or multiple binding domains that are directly or allosterically detected in $[^3H]$BTX-B binding assays (*30*).

Table II. Effects of *N*-Alkylamides on $[^3H]$BTX-B Binding and Sodium Uptake

| | $[^3H]$BTX-B Binding[a] | | Sodium Uptake[b] | |
|---|---|---|---|---|
| | $K_i$, µM | Hill Slope | $K_{0.5}$ | $E_{max}$ |
| BTG 502[c] | 1.43 | 0.83 | 1.7 | 1.17 |
| A | 45.8 | 1.04 | 13.7 (0.03)[d] | 0.43 |
| B | 50.1 | 0.75 | 16.0 (0.06) | 0.35 |
| C | 13.3 | 1.46 | 6.58 | 0.78 |
| D | 9.22 | 1.08 | 1.41 (0.02) | 1.10 |
| E | 21.6 | 1.07 | 1.99 | 0.37 |
| F | 244 | 0.28* | ND[e] | ND |
| G | 15.5 | 0.87 | ----[f] | ---- |
| H | > 60 | ND | ---- | ---- |
| I | > 60 | ND | ---- | ---- |

SOURCE: Adapted from ref. 24.

[a] Data from inhibition experiments were analyzed by least squares regression. The only Hill value statistically different from unity was that measured for F (*, Student's t-test, $p < 0.05$).

[b] Values for $K_{0.5}$, (µM) and $E_{max}$, (nmoles/assay) were analyzed by the method of Wilkinson (*29*).

[c] Data for BTG 502 are from (*23*).

[d] For compounds producing biphasic response curves, $K_{0.5}$ values for the high affinity component of sodium uptake, estimated by visual inspection of dose-response plots, are shown in parentheses.

[e] Value not determined.

[f] Stimulation of sodium uptake was not statistically significant.

**Effects of *N*-Alkylamides on Sodium Uptake.** Preliminary experiments were undertaken to establish the actions of BTG 502 (30 µM) both alone and in the presence of other sodium channel-directed toxins (Figure 6). Two major effects were measured in these tests: BTG 502 produced a 2.3-fold stimulation of uptake in the presence of ScV, and, in the absence of ScV, inhibited sodium uptake stimulated by the site 2 activators VTD (100 µM) and BTX (1 µM). DTM did not modify levels of uptake stimulated by BTG 502 measured in the absence of presence of ScV

In dose-response experiments, BTG 502 was found to be a potent inhibitor of activation of sodium uptake by BTX ($K_i$ of 2 µM) and, at a high concentration (100 µM), produced nearly complete inhibition of BTX-dependent uptake in the absence of ScV (data not shown). In parallel tests in which saturating concentrations of ScV were included in incubations with BTG 502, a similar potency for inhibition was measured (1.5 µM, Figure 7) but incomplete levels of inhibition were detected. The residual level of BTX-dependent sodium uptake observed in the presence of maximally effective concentrations of BTG 502 was equal to that produced by this compound in the presence of ScV, but in the absence of BTX.

These data provide evidence that BTG 502 acts as a partial agonist with respect to BTX at site 2 of the sodium channel. However, these findings also establish

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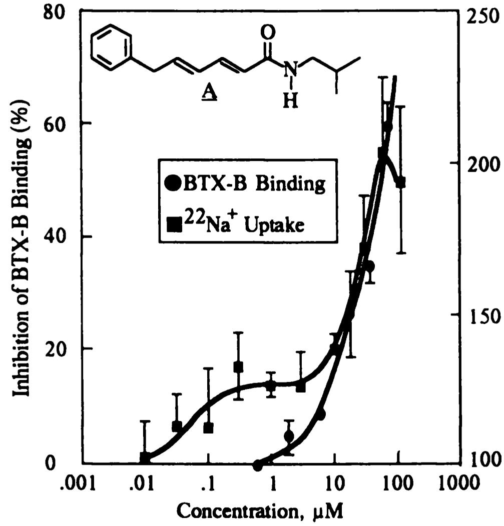

Figure 5. Concentration-dependent inhibition of $[^3H]$BTX-B binding (circles) and activation of sodium uptake (squares) by A. (Reproduced from ref. 24. Copyright 1990 American Chemical Society).

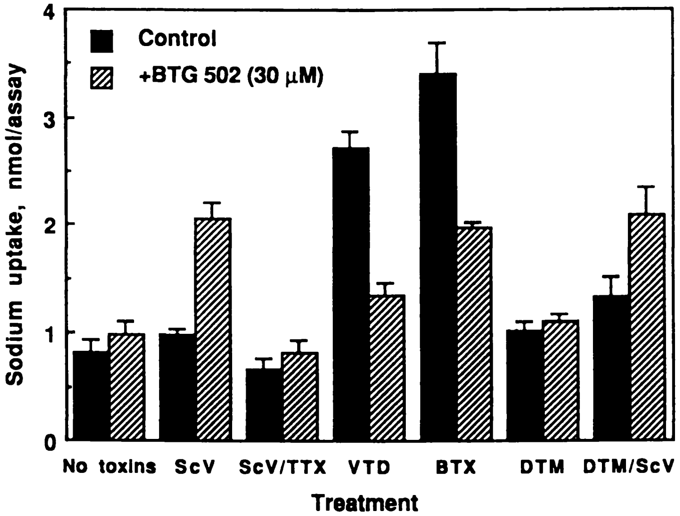

Figure 6. The effect of sodium channel neurotoxins [ScV (25 μg), TTX (10 μM), VTD (100 μM), BTX (1 μM), and DTM (10 μM)] on total sodium uptake in the absence (solid squares) or presence (hatched squares) of BTG 502 (30 μM). (Reproduced with permission from ref. 23. Copyright 1989 The American Society for Pharmacology and Experimental Therapeutics).

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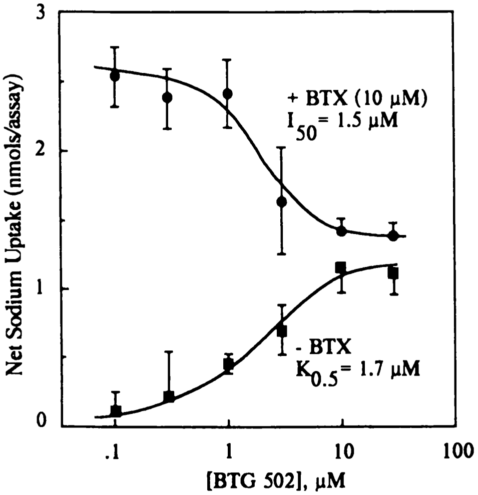

Figure 7. Concentration-dependent activation of sodium uptake (squares) and inhibition of BTX-dependent sodium uptake (circles) by BTG 502. (Reproduced with permission from ref. 23. Copyright 1989 The American Society for Pharmacology and Experimental Therapeutics).

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differences between *N*-alkylamides and other sodium channel activators. In assays with mammalian brain preparations, the potency of site 2 activators is typically enhanced by site 3 neurotoxins such as ScV (*19, 27*). With BTG 502, insignificant differences in potency were found for inhibition of BTX binding measured in the absence and presence of ScV. In addition, BTG 502 differed from other site 2 neurotoxins in that it failed to exhibit allosteric interactions with the pyrethroid insecticide DTM.

Whereas all of the compounds inhibited the binding of [$^3$H]BTX-B, only insecticidal *N*-alkylamides (BTG 502, **A-F**) were able to stimulate significantly the influx of sodium in the presence of a saturating concentration of ScV (Figure 3). The greatest values for stimulation of sodium uptake (approaching or exceeding 200% of control values) were measured in incubations with **A-D** (60 µM). In the absence of ScV, no statistically significant stimulation of uptake was detected with any of the analogues tested (data not shown).

Concentration-response parameters for the action of *N*-alkylamides as stimulators of sodium uptake are summarized in Table II. In assays with most of these analogues, maximal levels of sodium uptake were detected at concentrations that were subsaturating with respect to the inhibition of [$^3$H]BTX-B binding (Figure 4). Thus, the analogue concentrations producing half-maximal levels of enhancement ($K_{0.5}$) were generally less than the corresponding binding affinity constants for inhibition ($K_i$).

Of the compounds tested, **D** was the most potent ($K_{0.5}$= 1.41 µM) and efficacious ($E_{max}$= 1.10 nmoles/assay) activator of sodium uptake. Compound **F**, the analogue least potent as an inhibitor of [$^3$H]BTX-B binding, was also the least active stimulator of sodium flux. The low levels of stimulation measured in the presence of this analogue precluded the estimation of values for $K_{0.5}$ and $E_{max}$. No significant enhancement of sodium uptake was detectable in tests with **G**, **H**, or **I**. In addition, the stimulation of uptake produced by a sub-saturating concentration of BTG 502 (10 µM) was inhibited 54% by **G** (75 µM), suggesting that the analogues which were inactive as stimulators of uptake acted as antagonists (data not shown).

Two distinct patterns of stimulation were measured in dose-response experiments for the *N*-alkylamides that enhanced sodium uptake. The relationship between increasing concentration and enhancement of sodium uptake was monophasic in tests with BTG 502 (Figure 5), **C** (Figure 6) and **E** (data not shown). In experiments with these compounds, stimulation of uptake occurred within the same range of concentrations producing inhibition of [$^3$H]BTX-B binding. In contrast, biphasic patterns of stimulation were seen in experiments with compounds **A** (Figure 5), **B** and **D** (data not shown).

In tests with analogues producing biphasic uptake curves, inhibition of [$^3$H]BTX-B binding was measurable only at concentrations corresponding to the lower affinity component for the stimulation of sodium uptake (Figure 5). Because PTX-B stimulates sodium uptake in the presence of ScV via an action independent of site 2 (*21*), the possible actions of the analogues at the PTX-B recognition site were explored by assessing the effects of an *N*-alkylamide exhibiting biphasic stimulation of sodium uptake on sodium uptake stimulated by PTX-B. **A** at 0.3 or 75 µM did not inhibit PTX-B (10 µM)-dependent uptake, and the level of uptake measured in assays with combinations of **A** and PTX-B was equivalent to the sum of that produced by the two compounds individually (data not shown). In these experiments, stimulation of uptake was detected only in the presence of ScV and was blocked completely by TTX. Therefore, the high affinity component of sodium uptake resulted from the interaction between these compounds and the sodium channel at a domain other than site 2, rather than from an indirect effect on vesicle depolarization leading to a stimulation of sodium influx. The potential for exploitation of this binding site for insect control is unknown; however, for the *N*-alkylamides tested here, the stimulation of sodium uptake by this mechanism does not appear to be correlated with insecticidal activity (*9, 25*).

**Structure-Activity Relationships.** Although the series of compounds examined was somewhat limited, these studies permit the identification of some features of chemical structure that define the activity of the *N*-alkylamides as both effectors of sodium channel function and insecticides. The 2,4-dienamides **A-F** exhibited insecticidal activity and stimulated the uptake of radiosodium. In contrast, **H**, the 3,5-dienamide otherwise identical in structure to **B**, was neither toxic to insects nor active as an enhancer of sodium uptake. These findings confirm the critical role in this series of compounds for the position of unsaturation in effecting toxicity (*12, 25*) and implies that this requirement reflects the specificity of the target site. In addition, alterations in the length of the alkylene chain in *N*-(2-methylpropyl)-2,4-dienamides was found to influence the toxicity of these compounds to house flies and mustard beetles (*12, 25*). Results from sodium flux and [$^3$H]BTX-B binding assays showed that increasing the length of the chain by two carbons (compare **A** and **G**, Table II) effectively separates the binding of *N*-alkylamides to site 2 from the subsequent transduction event that alters sodium uptake. Thus, **G** apparently binds to site 2 but functions only as an antagonist of other activators and is not effective as an insecticide. This finding illustrates the need for functional assays as well as binding assays to establish the mechanisms of action of new compounds. Finally, methyl substitution at the α position of the amide moiety does not affect the activity of *N*-alkylamides as sodium channel toxins (see **A** and **B**, Table II) or as insecticides (*25*).

## Summary

The results of assays to determine the biochemical effects of *N*-alkylamides confirm previous findings from electrophysiological studies with insect nerve preparations (*13, 14*) and implicate site 2 of the sodium channel as the site of action for these compounds. Because the *N*-alkylamides appear to affect a sodium channel domain distinct from that of the pyrethroids and DDT, the development of such compounds represents an attractive strategy for the control of insect populations in which pyrethroid resistance has arisen.

## Acknowledgments

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# Chapter 23

# Phytochemical Antagonism of γ-Aminobutyric Acid Based Resistances in *Diabrotica*

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Susceptibility in adult western corn rootworm to chlorinated cyclodienes, γ-aminobutyric acid ($GABA_A$)-gated chloride channel antagonists, is increased seven-fold by short-term floral feeding on cultivated sunflower. Therein are strongly antifeedant germacranolide lactone angelates with picrotoxinin-like chloride ionophore neurotoxicity. GABA antagonists including picrotoxinin and bicuculline and methylenedioxyphenyl cytochrome P450 inhibitors like piperonyl butoxide and piperine are also strong rootworm feeding deterrents and toxicants. Molecular modeling indicates that a picrotoxinin-like receptor mediates antifeedant and internal effects of sunflower epoxysesquiterpene lactones. A bicuculline site for this and similar methylenedioxyphenyl compounds and a steroidal cucurbitacin site on the $GABA_A$-complex is also implied. GABA-dependent ionophores may mediate sensillar transduction of gustatory behavior, and thereby impact food habits that select for decreased resistance in *Diabrotica* species to cyclodiene epoxides. Compelling is the possibility that genes of both chemosensory and central GABA receptors are coordinately expressed.

Behavioral mechanisms for insecticide resistance in insects are much less studied than the physiological, biochemical or molecular basis of resistances to post-ingested or applied toxicants. Sparks et al. (*1* and refs. therein) concluded from their reviews of this subject that "behavior is observable physiology", and coselection of both a behavioral and an internal physiological mechanism of resistance should occur as readily as development of two separate physiological resistance mechanisms. Indeed, behavioral avoidance of non-host chemicals is a major route by which insects select foods. Although much less is known on the insect repellency of insecticides at sublethal dosages, the phenomenon has been recognized since synthetic insecticides were introduced (early studies reviewed in *2*). The molecular basis for action of chemical deterrents on both gustatory and olfactory sensory systems in insects is unknown.

Among plant anti-herbivore chemistry, a strong link does not exist between feeding deterrency and internal toxicity in insects (*3*), suggesting that behavioral rejection is not an adaptation to ingested effects but more an outcome of deterrent

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receptors with wide chemical sensitivity. Nevertheless, the coexpression of avoidance with toxicity-reducing mechanisms (detoxification, target site insensitivity, transport barriers etc.) should synergistically increase resistance to an otherwise toxic chemical. This may mask the incidence of positive correlations between antifeedant and toxicity activities; moreover, negative correlations would be expected if intensive selection to a particular insecticidal class of chemicals has already occurred. While within-generation phytochemical induction or inhibition of detoxification enzymes due to dietary shifts (i.e. intermittent chemical selection) is well-established in insects (*4-6*), the inheritability of this propensity as a resistance trait or its consequence on insecticide cross-resistance in subsequent generations is unclear.

Many insects, especially less polyphagous species, are fastidious feeders, and will starve themselves to death in presence of nutritionally-balanced diets unless key visual, olfactory, gustatory or mechanical cues are apparent in the food (*7*). This is particularly evident among the leaf beetles (family Chrysomelidae), the second largest family of herbivorous species in the animal kingdom (*8*). The species richness and success of leaf beetles as herbivores, many of which are host specific, are dependent on the development of strategies to coadapt with the numerous defensive chemicals in the plant kingdom. Chemoperception of toxic doses of these allelochemicals is an efficient means to overcome their presence (*9*), whereas a major post-ingestion mechanism that enables the utilization of otherwise poisonous food is enzymatic detoxification (*10*). Despite a plethora of study, key global food pests such as the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) and corn rootworms (*Diabrotica* spp.) remain without an adequate artificial substitute for food plants in multi-generational rearing. While this hampers efforts to follow inheritance of attributes conferring insecticide resistance, it indicates that control of insect feeding behavior is a viable strategy for management of these chrysomelids. Identification of key neural mechanisms responsible for this "starvation effect" may allow the chemical disablement of chrysomelid feeding.

## Corn Rootworm, Cyclodiene Resistance and Alternative Host Plants

*Diabrotica* leaf beetles (Chrysomelidae) are major Pan-American pests on corn, of which western corn rootworm, *D. virgifera virgifera* LeConte (WCR), is the most pestiferous. Recently established populations of WCR in central Pennsylvania, even in absence of selection pressure from chlorinated cyclodiene insecticides in the field for at least 15 generations, are greater than 300 times more resistant to aldrin (Table I) than an

**Table I. Susceptibility of Adult Corn Rootworm Populations to Neurotoxicants**

| | Topical $LD_{50}$ (μg/g insect)$^a$ | | | | | | |
|---|---|---|---|---|---|---|---|
| Rootworm Strain/ Species$^b$ | $GABA_A$ Chloride Channel Ligands | | | Acetylcholinesterase Inhibitors | | | Sodium Gate |
| | Aldrin | Picrotoxinin | Avermectin | Carbofuran | Terbufos | Isofenphos | Tefluthrin |
| Pa WCR | 1980 | 7850<br>111$^c$ | 328<br>58$^c$ | 1.16 | 2.91 | 3.39 | 1.0 |
| Pa NCR | 6.0 | 18,200<br>26.2$^c$ | 121<br>24$^c$ | 1.05 | 2.78 | 4.58 | 0.9 |
| Nd WCR | 14 | — | — | — | — | — | — |

$^a$50% mortality determinations at 24 hr by probit analysis.

$^b$Pa = Centre Co., PA field strain; Nd = non-diapause lab strain, French Agric. Res., Lamberton, MN

$^c$Estimated by injection; see ref. (*11*) for additional details.

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endemic population of northern corn rootworm, *D. barberi* Smith & Lawrence (NCR). Both of these univoltine rootworm species had formerly acquired high cyclodiene resistance prior to cancellation of these insecticides for rootworm control (*12*). Wild NCR populations presently are variably resistant to aldrin (*13*) whereas uniformly high WCR resistance remains in the field. To our knowledge, only the inbred, non-diapause laboratory strain of WCR (Table I) originally selected at the USDA Northern Grain Insects Research Laboratory is susceptible to cyclodienes. Today, larval rootworm control in corn is highly dependent on non-selective, soil insecticides, the bulk of which are carbamoyl and organophosphoryl inhibitors of nerve acetylcholinesterase. Development in rootworm of a broadly insensitive cholinesterase to organophosphates and carbamates would impair its chemical control. This scenario is emerging for another major chrysomelid pest, the multivoltine Colorado potato beetle (*14*).

Both WCR and NCR in Central PA remain equally susceptible to the acetylcholinesterase inhibitors carbofuran, terbufos and isofenphos (Table I). Also, no cross-resistance between aldrin and tefluthrin was evident, with high susceptibility (24 hr topical $LD_{50}$s around 1 µg/g insect, Table I) to this pyrethroid being found in both the aldrin susceptible and resistant species.

Resistance to aldrin in adult WCR , depending on food choice, can range 90 to 1200 times relative to NCR. Particularly noteworthy is that WCR reject flowers of the Asteraceae (= Compositae) that are readily acceptable to NCR. Present within these latter foods are aldrin resistance- (*15*) and longevity- reducing factors (*16*) and antifeedants (*17*) for WCR. Thus, susceptibility of WCR to aldrin increased seven times and longevity was reduced 40% when adults were maintained on sunflower (*Helianthus annuus* L.) inflorescences rather than corn, whereas NCR was equally susceptible to aldrin and exhibited no significant longevity reduction on this diet. From sunflower were isolated antifeedant sesquiterpene lactone angelates (see below), which coincidently may also exert the anti-resistance and -growth effects. The sunflower diet causes in WCR a 2-fold increase of aldrin epoxidation without an induction of epoxide hydration which would increase aldrin toxification to dieldrin without a parallel enhancement of its detoxification to the diol; by contrast a more coordinate induction of these enzymes occurs in NCR (*15*). Altered enzymatic detoxification pathways can explain diet-induced changes in aldrin susceptibility within adult life of rootworms; however, can reduced selection of the more corn-specializing WCR in comparison to NCR on Asteraceae foods retard resistance decay in the former species in absence of cyclodiene selection? More multi-generation study is necessary to determine if fewer progeny exhibiting cyclodiene resistance would result from female WCR adults that are challenged with Asteraceae diets. Sesquiterpene lactone antifeedants would be a likely chemical basis for this antagonism of cyclodiene resistance.

Early work by Chio and Metcalf (*18*) did not identify marked detoxification differences between NCR and WCR that alone could explain a large divergence in cyclodiene susceptibility (*19*). In other insect species, cyclodiene resistance has most often been associated with target site insensitivity (*20-21*). Since chlorinated cyclodienes act at the same type A γ-aminobutyric acid ($GABA_A$)-regulated chloride ionophore site as picrotoxinin, reduced sensitivity to picrotoxinin is strong evidence that receptor modification is mediating resistance to this class of insecticides (*22-23*). For WCR, a low cross-resistance between this plant neuroexcitant and cyclodienes was found, but the 4-fold between species resistance ratio for picrotoxinin was two orders of magnitude less than for aldrin (*24*; Table I) indicating that an insensitive GABA site was not the sole basis for cyclodiene resistance. We proposed that the high aldrin resistance of WCR is due to the joint actions of reduced alternative host plant feeding, 40% decreased steady-state penetration, 40% increased excretion, 40% increased detoxification at the nerve site and a four-fold nerve insensitivity to picrotoxinin in comparison to susceptible NCR (*24*).

## Terpenoid Regulators of Chrysomelid Herbivory: GABA Associations

Insecticidal and antifeedant sesquiterpenoids, many of which are epoxides (*25-31*), are major barriers to insect herbivory. Inhibitory cyclic sesquiterpenes for phytophagous insects have been identified from at least 28 genera of the terpenoid-rich Asteraceae (refs. cited in *11*). Germacranolides from inflorescences of cultivated sunflower have recently been associated with rejection by WCR of this marginal host plant (*17*).

Nortriterpenoidal cucurbitacins are responsible for the high prevalence of *Diabrotica* spp. feeding on bitter squash species (*32-33*). Among the most potent of known insect feeding stimulants, cucurbitacins are particularly abundant within the anther and filament of *Cucurbita maxima*, a much preferred squash species for corn rootworms as a pollen-source of food (*34*). Interestingly, for other chrysomelids such as the Cruciferae leaf beetles (*35*) and Colorado potato beetle (*36*), these "bitter-tasting" compounds are strong feeding deterrents.

By utilizing a flower disk from a bitter *Cucurbita maxima* species as a food matrix, we developed a consumption bioassay for rootworm to detect highly active antifeedants that counteract the potent feeding stimulation by cucurbitacins over a 48 hr period (*17*). Guided by this bioassay, a systematic fractionation of cultivated sunflower inflorescences led to isolation of more than 60 ethyl acetate-soluble principles of which only the germacranolide epoxides argophyllins A (**1**) and B (**2**) were strongly antifeedant for adult WCR (Table II). Isolation and identification was achieved by silica gel and Toyopearl TSK HW-40F chromatography followed by UV, $^1$H- and $^{13}$C-NMR and EIMS. Feeding deterrency decreased in order of sesquiterpenes >> diterpenes > flavonoids > dicaffeoylquinic acids (*17*).

Structure-activity comparisons of the twelve sesquiterpenes (**1**, **2**, **4-13**) isolated from cultivated sunflower (Table II) indicate that the 1,10-epoxy function (**1**, **2**) is most critical for potency of the feeding deterrent. A related germacranolide epoxide, argophyllone B (**3**), isolated from the wild sunflower, *H. argophyllus* (*37*), is likewise strongly antifeedant to WCR (Table II). All active terpenoids for WCR were at least bicyclic, moderately polar, and if not epoxides, contained at least one electrophilic center such as an α, ß-unsaturated lactone or -ester, or a ketone or olefin with extended conjugation. The lactone and angelyloxy groups were, by themselves, not essential to activity based on the strong activities of the eudesmanes **13** and santonin **14**, respectively. The electrophilic groupings, along with the general polyoxygenation, may maintain the correct overall molecular polarity to allow interaction with the taste receptor. The latter sesquiterpene, α-santonin, a known insect antifeedant and anti-helminthic (*30*), is of interest from its high activity in comparison to the poorly active conjugated ketone **11** of the germacranolide angelate series. A comparable 1, 2-epoxyeudesmanolide to that of argophyllin A or argophyllone B may expectedly be highly active on WCR. The 3,4-dihydroxy or 15-hydroxy-3-oxo groupings conferred higher antifeedant effect than 3, 15-hydroxylation alone (**1**, **3** vs **2**). 10-O-Methylation had minimal effects on activity of the free hydroxy analogue. The unexpected strong activity of **4** is probably due to an unknown epoxide contaminant (Table II). Polycyclic sesquiterpene lactones with unusual ring types including the endoperoxide artemisinin **15** , an anti-protozoan (*38*), and picrotoxinin **16** (see below) are also good antifeedants for WCR (Table II), but the ant-repelling caryophyllene oxide **17** (*39*) was not.

Limonin (**18**), a potent antifeedant for another chrysomelid, the Colorado potato beetle (*40*), is only moderately effective on WCR and interestingly gives a weak stimulatory effect at high dose (Table II). The most reputed of these tetranortriterpene limonoids for its wide feeding deterrency among insects, azadirachtin **19** (*41-44*), is strongly antifeedant for adult southern corn rootworm, *Diabrotica undecimpunctata howardi* Barber (*45*) and has recently been found toxic for larval WCR (*46*).

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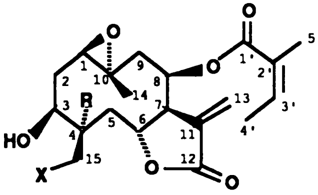
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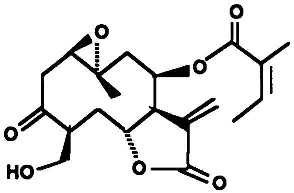
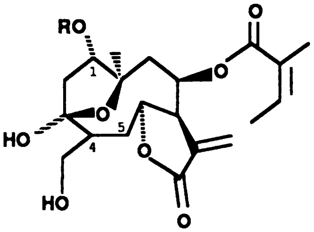
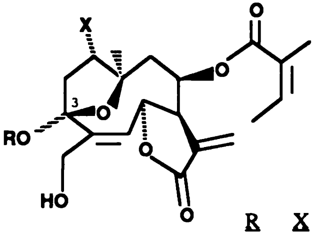
| 1 Argophyllin A | OH | H |
| 2 Argophyllin B | H | OH |



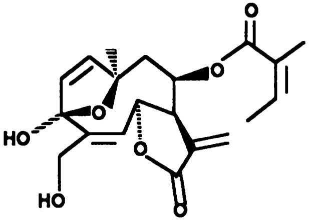
3 Argophyllone B



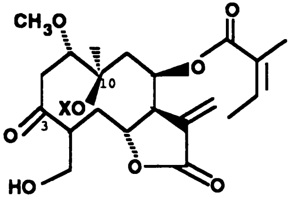
| | R |
|---|---|
| 4 4,5-Dihydroniveusin A | H |
| 5 1-O-Methyl-4,5-dihydroniveusin A | CH3 |



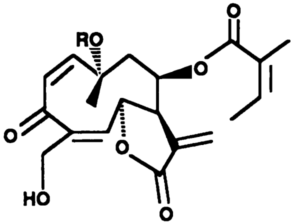
| | R | X |
|---|---|---|
| 6 3-O-Methylniveusin A | CH3 | OH |
| 7 Niveusin B | H | H |



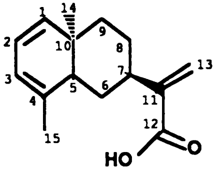
8 1,2-Anhydridoniveusin A


$CH_3O$
XO
10
3
HO
O

| | X |
|---|---|
| 9 3-Oxo form of 5 | H |
| 10 10-O-Methyl- ether of 9 | CH3 |



| | R |
|---|---|
| 11 15-OH-3-dehydrodesoxytifruticin | H |
| 12 10-O-Methyl- ether of 11 | CH3 |


1
2
3
4
5
6
7
8
9
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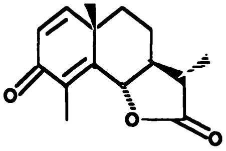
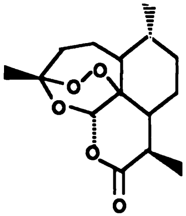
11
12
13
14
15
HO
O

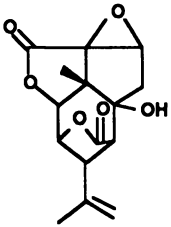
13 12-Carboxyeudesma-1,3,11(13)-triene



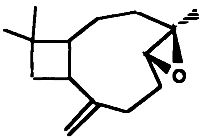
14 alpha-Santonin



15 Artemisinin



16 Picrotoxinin



17 Caryophyllene oxide

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**Table II. Sesquiterpene Antifeedants for Adult Western Corn Rootworm[a]**

| TYPE<br>Structure No. | compound name | Relative Consumption (treated/control) X 100[b] | | | | | |
|---|---|---|---|---|---|---|---|
| | | 40 µg/disk | | | 80 µg/disk | | |
| | | 5 hr | 24 hr | 48 hr | 5 hr | 24 hr | 48 hr |
| HELIANTHUS | | | | | | | |
| 1 | argophyllin A[c] | 32 ± 6 | 23 ± 4 | 24 ± 1 | — | — | — |
| 3 | argophyllone B[d] | 14 ± 2 | 27 ± 7 | 30 ± 8 | — | — | — |
| 2 | argophyllin B | 69 ± 7 | 52 ± 4 | 75 ± 5 | — | — | — |
| 4 | 4,5-dihydroniveusin A+ epoxide[e] | 33 ± 7 | 58 ± 6 | 90 ± 11 | 58 ± 17 | 39 ± 12 | 67 ± 8 |
| 6 | 3-O-methylniveusin A[c] | 62 ± 10 | 75 ± 8 | 89 ± 1 | 43 ± 9 | 30 ± 9 | 65 ± 5 |
| 7 | niveusin B + **8** [e] | 50 ± 14 | 82 ± 4 | 88 ± 3 | 40 ± 10 | 49 ± 4 | 56 ± 7 |
| 13 | 12-carboxyeudesma-1,3,11-triene | 63 ± 8 | 55 ± 6 | — | 43 ± 11 | 75 ± 8 | — |
| 9 | 3-oxo-10-hydroxy derivative of 5[c] | — | — | — | 30 ± 7 | 64 ± 9 | 72 ± 6 |
| 10 | 10-O-methyl ether of 9[c] | 47 ± 8 | 80 ± 5 | 94 ± 3 | 49 ± 8 | 64 ± 2 | 76 ± 5 |
| 12 | 10-O-methyl ether of 11 | 57 ± 6 | 69 ± 3 | — | 36 ± 10 | 68 ± 12 | — |
| 5 | 1-O-methyl-4,5-dihydroniveusin A[c] | 87 ± 9 | 97 ± 9 | 98 ± 1 | 63 ± 8 | 71 ± 2 | 78 ± 4 |
| 11 | 15-OH-3-dehydrodesoxytifruticin[c] | 55 ± 10 | 97 ± 16 | 90 ± 6 | 68 ± 36 | 75 ± 8 | 82 ± 7 |
| OTHER SESQUITERPENES | | | | | | | |
| 14 | santonin | 21 ± 7 | 41 ± 7 | 61 ± 5 | 20 ± 11 | 40 ± 5 | 56 ± 9 |
| 15 | artemisinin | 32 ± 5 | 61 ± 13 | 79 ± 6 | 65 ± 26 | 37 ± 13 | 55 ± 6 |
| 16 | picrotoxinin | 48 ± 17 | 69 ± 9 | 88 ± 5 | 48 ± 21 | 73 ± 14 | 82 ± 9 |
| 17 | caryophyllene oxide | 68 ± 33 | 98 ± 4 | — | 28 ± 24 | 94 ± 2 | — |
| *LEPTINOTARSA* ANTIFEEDANT | | | | | | | |
| 18 | limonin (nortriterpenoid) | 22 ± 5 | 64 ± 5 | 90 ± 3 | 342 ± 260 | 122 ± 18 | 109 ± 8 |

[a] Dual choice tests between terpenoid-treated and solvent control using bitter squash disk bioassay.

[b] Mean ± SEM (4); all terpenoids significantly antifeedant ($p < 0.05$) at 1 or more dose-time points.

[c] Five and 24 hr data from ref. (*17*).

[d] Kindly provided by Dr. Robert D. Stipanovic from USDA, ARS, College Station, TX.

[e] Compound **4** contained 30% of an unidentified epoxide; **7** had 12% 1,2-anhydridoniveusin A.

Sesquiterpene lactone antifeedants are expressed in cultivated sunflower in a remarkably efficient manner so as to defend the plant from insect herbivory. These epicuticular exudates of glandular trichomes (*47*) are most abundant in leaves at the apex of sunflower seedlings (*48*). Extending this work into reproductive stages of the plant, we have found that total loading of sesquiterpene lactone angelates (STLAs) is highest in leaves near the meristem and in disk flowers producing the ensuing generation, and the most active antifeedant for WCR, **1**, is the principle compound exuded (*49*). Based on high performance thin-layer chromatography (HPTLC) and UV reflectance densitometry with sensitivity down to 200 ng of applied sesquiterpene, total STLAs in order of organ abundance are leaf ~ disk flower >> bract > ray flower (Table III) with none found in achene, pollen, stem, or root. Argophyllin A predominated in upper (younger) leaves and all floral tissues followed generally in order of concentration by **11, 7, 2** and **4** (Figure 1); sesquiterpenes previously identified only from sunflower leaves (*50-51*). A good correlation occurred between argophyllin A content and antifeedant activity either on a tissue (Table III) or whole plant basis (Figure 1). Protection of the meristem and plant progeny are particularly evident. Thus argophyllin A and related STLAs from disk floret trichomes may effectively defend cultivated and wild *Helianthus* species against corn rootworm or other pollen and seed predators such as the sunflower moth (*52-54*).

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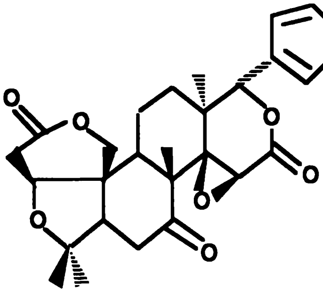
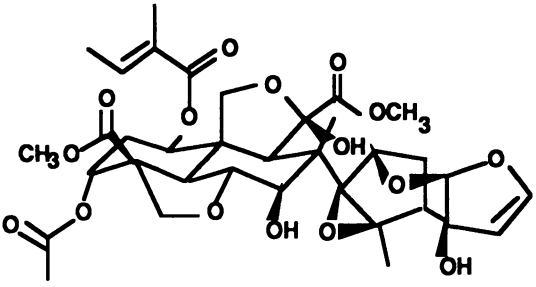


**18** Limonin



**19** Azadirachtin

## Table III. Association Between Argophyllin A Distribution in Sunflower Tissues and Rootworm Antifeedant Activity

| part[a] | Relative Consumption at 80 μg/disk (treated/control)[b] 24 hr | 48 hr | STLA Content by HPTLC (μg/g tissue)[c] Total | Argophyllin A |
|---|---|---|---|---|
| ray flower | 83 ± 9 | 97 ± 4 | 13 | 7 |
| flower bract | 67 ± 9 | 86 ± 5 | 20 | 12 |
| disk flower | 66 ± 8 | 84 ± 5 | 343 | 129 |
| leaf | 52 ± 3 | 60 ± 3 | 445 | 200 |

[a] Surface extracted with ethyl acetate; STLAs then adsorbed to silica gel and selectively co-eluted.
[b] Squash disk in choice bioassay treated with STLA-enriched eluate; mean ± SEM for 7-16 replicates.
[c] Based on scanning UV densitometry of HPTLC - resolved STLA.

Sunflower STLA antifeedants gave injected symptoms in adult WCR similar to **16** and cyclodiene insecticides, both GABA$_A$-chloride channel directed ligands, suggesting a link between sesquiterpene neurotoxicity and GABA (*17*). Picrotoxinin, a plant sesquiterpene epoxide lactone from tropical *Anamirta* spp., family Menispermaceae, antagonizes cyclodiene insecticide binding in mammals and other insect species (*22, 23, 55, 56*). Structural inspection of **1**, **16** and a cyclodiene epoxide such as dieldrin indicates a common bulky head group containing multiple electronegative atoms together with a narrow hydrophobic tail containing an electrophilic site. The compelling three-dimensional structural similarity between argophyllin A, picrotoxinin and dieldrin (Figure 2) suggests action through a shared picrotoxinin receptor site (*57, 58*), and led us to explore the role of *Helianthus* STLA and other GABA-directed chemistry in maintenance of cyclodiene resistance in the *Diabrotica*.

## GABA-Directed Actions in Insects

The GABA-dependent chloride channel is ligand-gated, and responds to a plethora of chemistry in both vertebrates (*59-61*) and insects (*62-64*). Inward chloride movement neutralizes nerve impulse propagation by decreasing the positive potential (activation)

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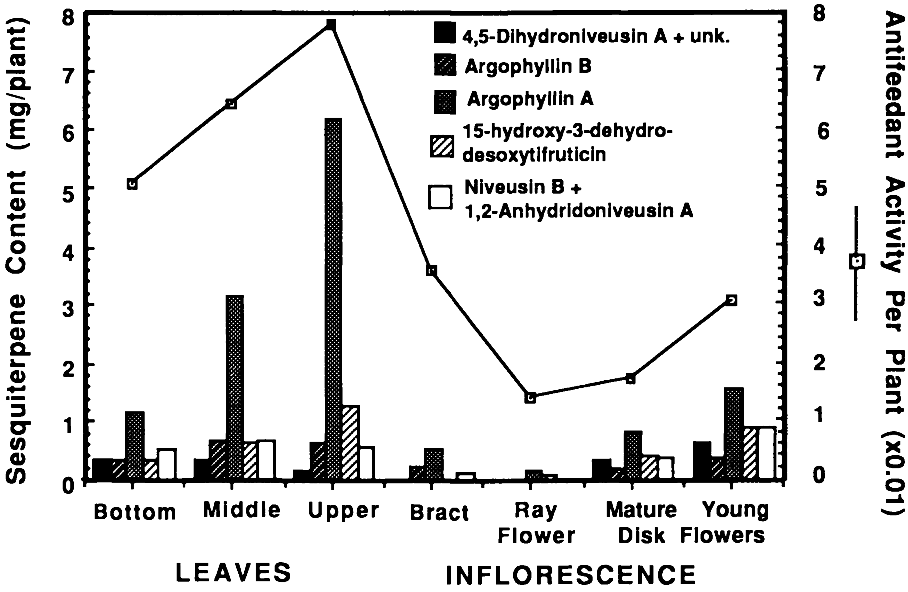

Figure 1. Relationship of STLA content, particularly argophyllin A, in various tissue extracts of flowering *Helianthus annuus* L. with total antifeedant activity on WCR (= extract mass ÷ mass in bioassay ÷ treated to control consumption ratio).

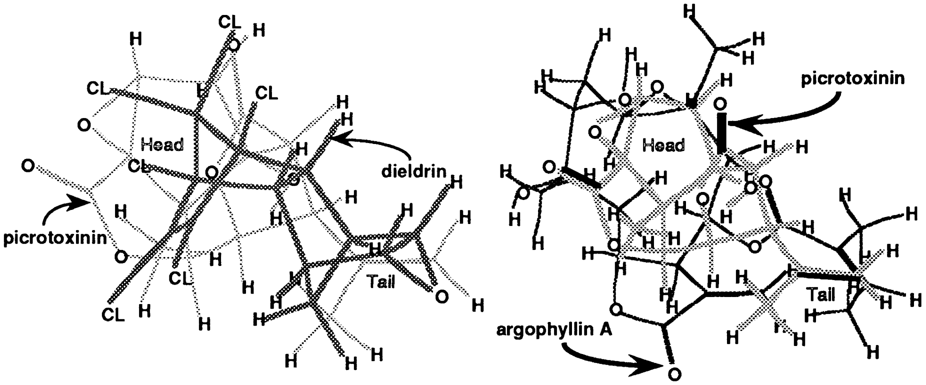

Figure 2. Molecular fit using Tripos Associates Alchemy II modeling program between picrotoxinin and dieldrin, and between picrotoxinin and argophyllin A.

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generated by inward movement of sodium through voltage-gated channels. The net effects of inhibitory and excitatory synapses will determine if a postsynaptic cell fires (or contracts, etc.). GABA functions at both peripheral (neuromuscular junctions etc.) and central synapses within the nervous system of insects, in contrast to mammals where it mediates primarily fast neuroinhibition in the central nervous system (CNS). These readily available peripheral sites make GABA targets particularly attractive for design of selective insecticides. The receptor site for neurotransmitter and direct GABA agonists (e.g. muscimol) and antagonists (e.g. bicuculline) is unique from binding sites of other classes of allosteric GABA antagonists (e.g. cyclodienes, picrotoxinin, cage convulsants) and potentiators (e.g. barbituates, benzodiazepines, analgesic steroids) which are located on the same channel complex. The insect GABA-gated chloride channel of the CNS has similar overall ligand sensitivity to that of the vertebrate except for a general insensitivity to bicuculline antagonism of GABA binding typical of vertebrate $GABA_A$ receptors (*62-64*). Allosteric antagonism of GABA binding at the chloride channel is the primary mechanism for cyclodiene neurotoxicity (*22, 23, 56, 62*). Trioxabicyclooctanes (*65, 66*) and related dioxatricycloalkenes (*67*), promising future insecticides, are potent allosteric antagonists that probably bind similarly to the picrotoxinin convulsant site on the chloride ionophore complex.

Extensive efforts have recently gone into commercialization of the fermentation products avermectins and milbemycins, a new class of chloride ionophore-directed pesticides. These 16-membered macrocyclic lactones stimulate chloride movement through both GABA-dependent and independent channels, and can give mixed agonistic and antagonisitc effects on GABA particularly in vertebrates (reviewed in *68*). In our studies, avermectins are two to three times less toxic to aldrin-resistant WCR than to susceptible NCR (Table I), indicating some cross-resistance between cyclodiene insecticides and avermectin. Mild cuticular barriers to avermectin penetration and extreme barriers to picrotoxinin penetration in these *Diabrotica* were found based on decreased topical versus injected susceptibilities (Table I). This contrasts with the ease of penetration of the more lipophilic aldrin (*24*), and suggests that moderate to low polarity is required for optimal transit of externally-applied $GABA_A$ directed agents to neuromuscular or central sites within adult corn rootworm.

**Relevance to Cyclodiene Resistance and Insect-Plant Associations.** The $GABA_A$ receptor is one of the only sites of action identified for epoxides. Chlorinated cyclodiene epoxides including dieldrin, endrin and heptachlor epoxide bind more tightly than their respective olefin to the picrotoxinin site on this receptor (*22, 23, 56, 62*). Cyclodiene resistant WCR avoid plants laden with epoxySTLA that potentially cross-react with an altered picrotoxinin site at a $GABA_A$-like receptor. By contrast, most wild NCR populations, although formerly resistant, are presently fully susceptible to cyclodienes, and readily consume Asteraceae containing epoxyterpenoids (see above; *17*). In turn, argophyllin A is less antifeedant for both NCR (not shown) and S-WCR than for R-WCR (Table IV), but both the cyclodiene-susceptible *Diabrotica* strains are more sensitive to picrotoxinin than R-WCR (Tables I, IV).

Compelling is the possibility that a $GABA_A$-like receptor is mediating taste perception in corn rootworms, and that external and internal effects of GABA-acting phytochemicals in food influence the maintenance of cyclodiene insecticide resistance. Thus, argophyllin A or other epoxySTLA selection on NCR may have altered both its peripheral and central GABA receptors to retard binding of this sterically-larger epoxySTLA in comparison to picrotoxinin (Figure 2), resulting in a restricted pocket that still accommodates picrotoxinin which it never naturally encounters. Such a pocket would also bind the smaller dieldrin and other cyclodienes that are no longer used or present in the rootworm environment. Similar selection by epoxySTLA in R-WCR may alter the picrotoxinin-site for more sensitivity to picrotoxinin and cyclodienes at the expense of the argophyllins. Behavioral avoidance of Asteraceae chemistry that impact

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**Table IV. Potency of GABA-Active (?) Antifeedants and Toxicants on Cyclodiene Resistant and Susceptible Western Corn Rootworm[a]**

| PUTATIVE SITE | Dose for 50% Reduced Consumption after 24 hr ($ED_{50}$)[b] | | | Topical 48 hr $LD_{50}$ (µg per insect)[c] | | |
|---|---|---|---|---|---|---|
| compound name | R-WCR | S-WCR | R/S | R-WCR | S-WCR | R/S |
| PICROTOXININ | | | | | | |
| picrotoxinin | 115 | 51.9 * | 2.22 | 9.6[d] | 7.4[d] | 1.3 |
| argophyllin A | 22.5 | 71.6 * | 0.32 | >10[d] | >10[d] | ---- |
| dieldrin | Toxic | Toxic | ---- | 12.0 | 0.24[e] | 50 |
| BICUCULLINE | | | | | | |
| bicuculline | 4.71 | 2.47 * | 1.91 | >10[d] | >10[d] | ---- |
| piperonyl butoxide | 7.20 | 13.4 | 0.54 | 5.79 | 11.5 | 0.50 |
| piperine | 36.7 | 31.2 | 1.18 | ~100 | > 150 | ---- |

[a] Dual choice tests in bitter squash disk bioassay with wild R and non-diapause S populations of WCR.

[b] ED50 in µg per disk determined by MINITAB using at least 4 dosages and 4 replicates per dose; significantly different populational response to chemical at $p < 0.05$ indicated by *.

[c] 50% mortality determinations by probit analysis after Abbott's correction.

[d] 48 Hr LD50 by injection in DMSO.

[e] For aldrin.

this major inhibitory neurotransmitter of insects may allow cyclodiene resistance to be maintained in WCR.

## GABA and Transduction of Chemosensory Information

**Feeding Inhibition.** Since picrotoxinin was both antifeedant and toxic to WCR, other established GABA ligands were screened on this beetle. (+)-Bicuculline was a potent antifeedant (Table IV), and as for picrotoxinin was more active on the cyclodiene-susceptible strains of *Diabrotica* than R-WCR (Figure 3). The $ED_{50}$ ratio for R-WCR to S-NCR was over 30-fold, whereas the sensitivity ratio between WCR strains was only 2-fold. All the known or putative GABA antagonists screened here with strong feeding deterrency were also acutely toxic to rootworms, although $ED_{50}$ levels were not always achieved at the highest dose tested (Table IV). The bicuculline activity was unexpected since most (reviewed in *63, 64*) but not all studies (*69, 70*) on internal GABA receptors in insects have indicated a bicuculline-insensitivity. However, external insect receptors may be more available to this tertiary aminoalkaloid and hence more characteristically $GABA_A$-like than internal or centrally-located sites. Conversely, dieldrin was inactive as an antifeedant at the highest dose presented to either S-WCR (0.4 µg/disk) or R-WCR (40 µg/disk) whereupon acute toxicity was observed (Table IV). This may be due to its hydrophobicity and non-availability for interaction via the aqueous phase with sensillar taste receptors on mouth palpi or tarsi of the beetle.

Stereospecificity requirements for antifeedant activity of bicuculline isomers and related phthalideisoquinoline alkaloids such as hydrastine (*71*) paralleled that reported for relative binding affinity or convulsant activity at the neurotransmitter site of the $GABA_A$ receptor (*72*). (+)-Bicuculline (1S, 9R), a bis-methylenedioxyphenyl (MDP)

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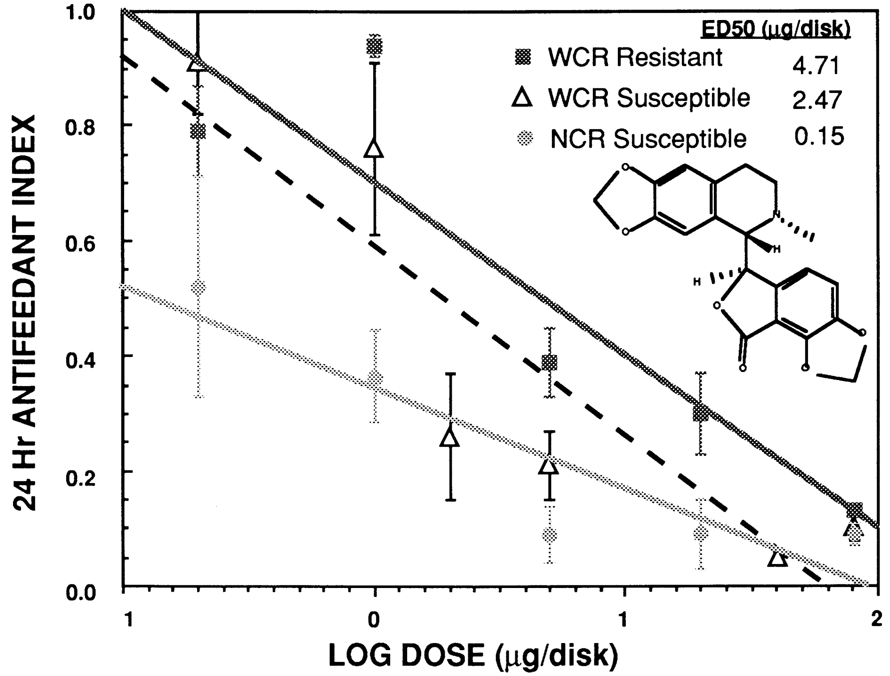

Figure 3. Antifeedant effect of (+)-bicuculline on cyclodiene-resistant and -susceptible corn rootworms.

isoquinoline from *Dicentra* spp. (Papaveraceae), was much more antifeedant than (-)-bicuculline (1R, 9S), and hydrastine was much more antifeedant than its truncated hydrastinine derivative (Figure 4). The conformation of (+)-bicuculline is more isosteric for the biologically active conformer of GABA than its (-)-isomer (*73*), and typically binds to the GABA$_A$ receptor with much higher affinity (*72, 74*). (-)-ß-Hydrastine, the open methylene-4',5'-dioxy analogue of bicuculline, has long been known as a vertebrate convulsant albeit less active than (+)-bicuculline but more active than its degradation product, hydrastinine (*75*), which lacks the phthalide ring. Recently, (+)-hydrastine was shown to be a 2-8 times more potent mammalian GABA$_A$ blocker than (+)-bicuculline (*76*). Other non-phthalide classes of isoquinoline alkaloids lacking (*77*) or containing the MDP group are antifeedant to lepidopterous insects, with MDP compounds consistently more toxic or repellent (*78*). The exciting possibility that a bicuculline-sensitive GABA receptor resides in peripheral sensory sites of insects is emerging.

Most interestingly, another MDP compound, piperonyl butoxide, an established inhibitor of cytochrome P450-mediated oxidation in WCR (*79*) and other insects (*80, 81*), was both highly antifeedant and unusually toxic to WCR (Table IV) with 96 hr topical LD$_{50}$s less than 2 µg per beetle. The feeding inhibition and latent toxicity may be associated with direct GABA antagonism in that piperonyl butoxide shares structural features common to bicuculline (Figure 5) with an electronegative ether atom instead of nitrogen at position 2 of the MDP side chain which also contains additional ether atoms that can freely configure with oxygen atoms of the phthalide ring of bicuculline.

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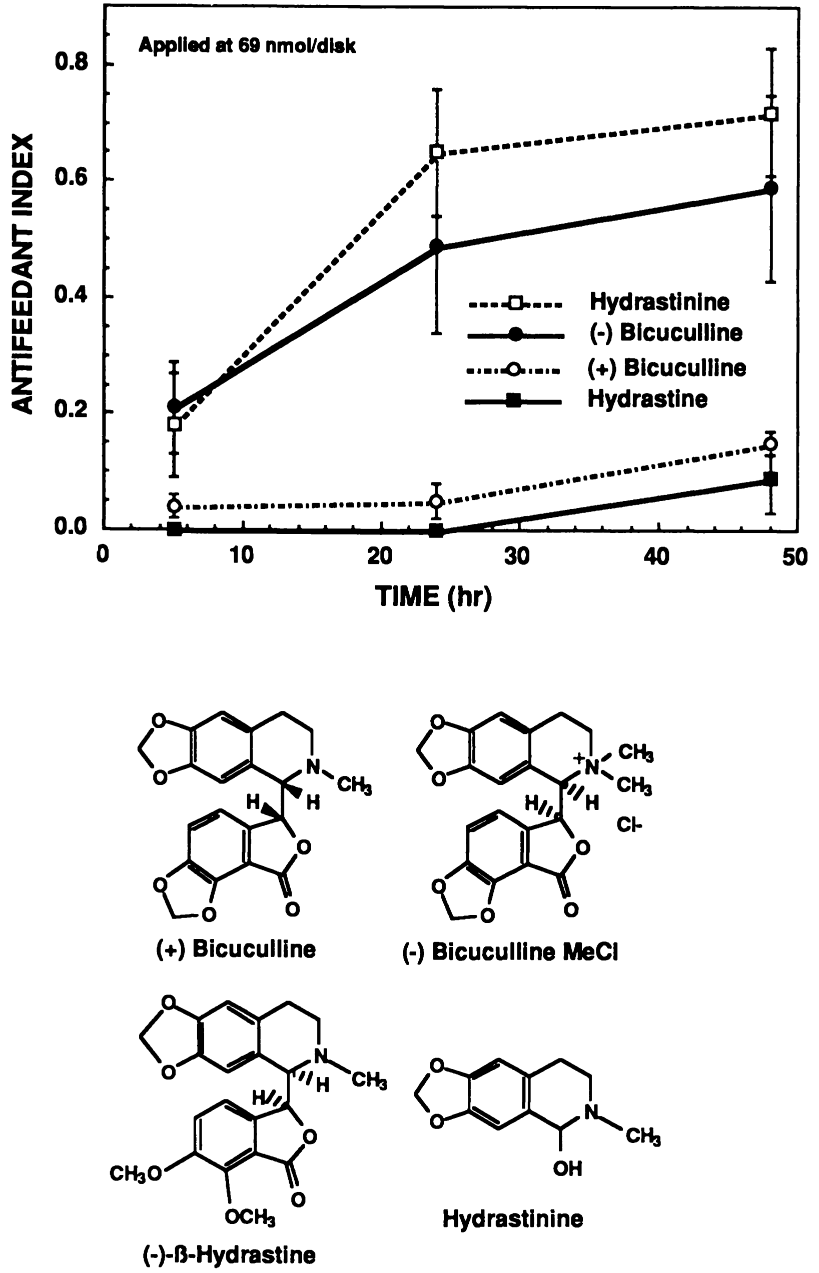

Figure 4. Feeding deterrency of bicuculline analogues to cyclodiene-resistant western corn rootworm.

Moreover, piperine from black and red peppers (Piperaceae), an MDP alkaloidal prototype of insecticidal N-isobutylamides also found in various Asteraceae and Rutaceae (*82*), was also antifeedant and clearly neurotoxic to WCR (Table IV), particularly if ingested. The side chain to the MDP of piperine, while electronically rich due to conjugation, is less sterically flexible than piperonyl butoxide (Figure 5). This may explain its poorer antifeedant activity and presumed lower ability to mimic bicuculline. Since piperine is structurally similar to N-isobutylamides now known to antagonize the action of voltage-sensitive sodium channels (*83*), it is likely this action is more important for internal neurotoxicity although feeding deterrency may arise from a bicuculline-sensitive ionophore. Like piperonyl butoxide, piperine inhibits cytochrome P450 (*84*). However, other known MDP insecticide synergists inhibiting this hemoprotein including safrole, isosafrole, and piperonyl isobutyrate which lack the long side chain of piperonyl butoxide and piperine were very weak antifeedants and toxicants for WCR (unpubl. data). MDP compounds are widespread in plants, mostly lacking overt toxicity (*85*) but having synergistic effects on more toxic co-occurring allelochemicals. Few have been screened for antifeedant activity. While inhibition in WCR of both internal and perhaps even peripheral cytochrome P450 detoxification of plant allelochemicals may explain MDP activities, alternative action as competitive GABA antagonists is indicated.

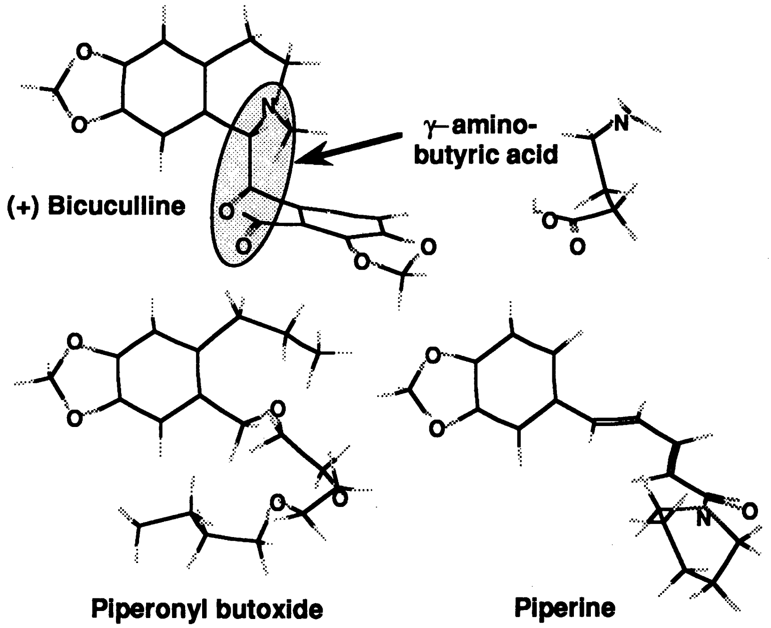

Figure 5. Comparison of conformational structures of (+)-bicuculline, piperonyl butoxide and piperine with the probable GABA binding pocket on the receptor.

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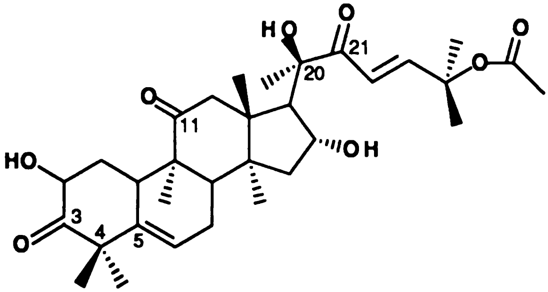
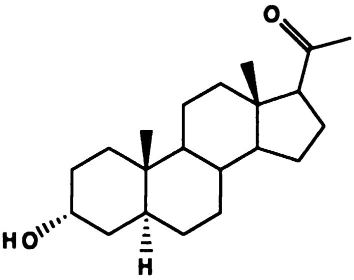
**A GABA$_A$ Receptor Model for Taste Chemoperception.** In WCR, gustatory feeding deterrents and GABA$_A$-chloride channel directed neurotoxicants share similar structural determinants. Our receptor model to explain mediation by GABA-dependent chloride ionophores of sensillar transduction of gustatory behavior is presented in Figure 6. Binding of picrotoxinin-like or bicuculline-like antagonists of chloride movement results in antifeedant behavior. Terpenoid epoxides like argophyllins and perhaps azadirachtin and citrus limonoids interact with an allosteric picrotoxinin-site. That argophyllin A and picrotoxinin share competitively the same taste receptor site is suggested by the inability of picrotoxinin at a dose up to 160 μg per disk to improve on the strong antifeedant effect of 60 μg per disk of argophyllin A in the WCR feeding bioassay (rel. consumption, treated to solvent control, at 24 hr of 23% ± 4% for argophyllin A; 20 ± 4% for argophyllin A plus picrotoxinin). Antagonism by bicuculline-like isoquinoline alkaloids and similar MDP compounds occurs through binding to the "neurotransmitter" site of the GABA-sensitive taste receptor. Perhaps the potent induction of feeding by cucurbitacins in adult (*32*) and larval (*86*) *Diabrotica* is also mediated by the same receptor. By analogy, cucurbitacin as an allosteric GABA agonist would facilitate chloride movement leading to a stimulatory behavior.

In support of this, analgesic steroids of similar structure to cucurbitacin B (**20**) such as 5α-pregan-3α-ol-20-one (**21**) are strong potentiators of vertebrate GABA$_A$-receptor mediated chloride movement (*87*) although less active in central nerve sites of the house fly (*88*). We found this pregnane steroid to antagonize the stimulatory action of cucurbitacins in the bitter squash disk bioassay resulting in feeding inhibition with an ED$_{50}$ of about 40 μg per disk. The 3-one of cucurbitacin B is orientated below the ring plane due to the 5-ene restriction of this unusual 4, 4-dimethyl-5-ene-11, 21-dione-20-ol lanosterol-like structure, and closely resembles the active conformation of 3α-ol pregnane or androstane steroids known to be GABA-acting anesthetics (*87, 89*). If cucurbitacins are acting via GABA, then the taste receptor site for this steroidal compound in *Diabrotica* is divergent from that of most insects and vertebrates where these compounds are markedly bitter and inhibitory to feeding (*32, 33*). Conceivably, the receptor may be specific to cucurbitacin to the extent that similar GABA-potentiating steroids for other organisms bind in an inhibitory fashion.

**Feeding Stimulation.** By this model, direct or allosteric GABA agonists should be stimulatory to *Diabrotica* feeding. In our bitter squash disk feeding bioassay, both GABA and muscimol, a direct GABA agonist, had no significant effect on WCR feeding at 80 μg per disk. However, at this dose GABA antagonized the antifeedant activity of 3 μg per disk of bicuculline (rel. consumption, treated to control, at 48 hr of



**20** Cucurbitacin B



**21** 5α-Pregnan-3α-ol-20-one

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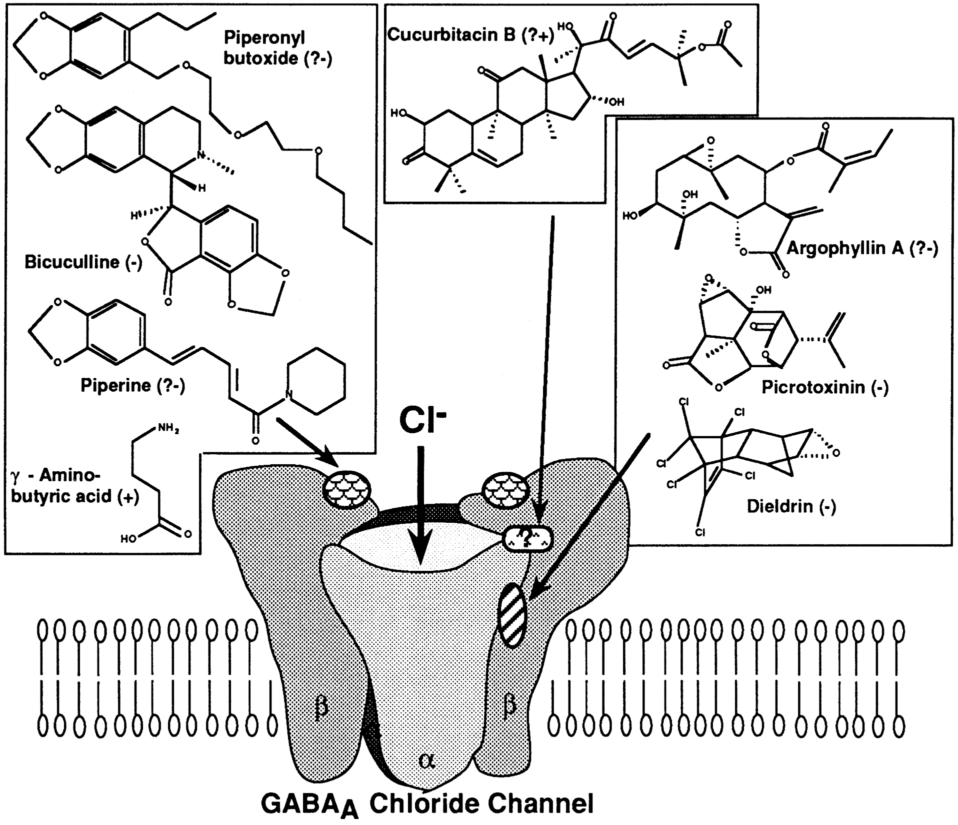
Piperonyl butoxide (?-)
Bicuculline (-)
Piperine (?-)
γ - Amino-butyric acid (+)
Cucurbitacin B (?+)
Argophyllin A (?-)
Picrotoxinin (-)
Dieldrin (-)
Cl⁻
β
α
β
?
GABA$_A$ Chloride Channel

Figure 6. GABA-dependent ionophore as a basis for taste chemoperception in chrysomelid beetles. Binding of antagonists (-) at "neurotransmitter" or allosteric sites gives antifeedant behavior whereas agonists (+) stimulate feeding. Putative modulators (?) include cucurbitacins that bind to a steroidal site on the chloride channel complex.

30% ± 8% for bicuculline; 61 ± 3% for bicuculline plus GABA ). Most likely, exogenous GABA and its agonists are unable to override the potent feeding stimulation by endogenous cucurbitacins within this adult WCR bioassay. Unfortunately, no other practical consumption bioassay is presently available for short-term feeding tests on adult WCR.

Colorado potato beetle is markedly stimulated to feed by GABA both in the larval (*90*) and adult (*91*) stages. GABA, L-alanine and sucrose stimulate the firing of the same cell of the α-galeal sensillum, but at least two separate receptors exist in this cell for sugars and for amino acids, respectively (*92*). While Mitchell (*93*) argues that the action of the non-protein amino acid GABA is a secondary adaptation to a specific L-alanine response based on structure-stimulation relationships (i.e. more flexible GABA folds into L-alanine active site), it is intriguing that this gustatory sensillum is so insensitive to other L-amino acids indicating a more specific GABA receptor may be involved. Alkaloidal feeding deterrents for this chrysomelid including the isoquinoline papaverine antagonize the GABA response (*92*), suggesting that net chemical interactions at peripheral gustatory receptors are determining the commitment to feed. It remains to be determined how general a GABA-stimulated galeal sensillum is among the Chrysomelidae. In this regard, only recently has evidence been obtained that GABA is a major vertebrate neurotransmitter in nerves that innervate taste buds based on work with the mudpuppy, *Necturus maculosus*, although a few earlier studies detected GABA action on rat and frog gustatory responses ( reviewed in *94*).

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**Molecular Interactions at Gustatory Sites.** Many antifeedants (*95, 96*) have electrophilic centers including allylic hemiketal, conjugated ketone, and epoxide sites in addition to the conjugate lactone which may interact with critical nucleophiles such as thiol (*97*) and amino groups (*98*) of sensory receptors. It remains to be determined if electrophilicity is associated with the GABA-like actions of terpenoid epoxides such as argophyllins, particularly the picrotoxinin-like effects.

Optimal polarity for interactions at an exterior chemosensory receptor are probably very different from that for GABA sites in the CNS or at neuromuscular junctions of insects. In the former, more polar, aqueous soluble neurotoxicants such as bicuculline, picrotoxinin, and argophyllins have a much better probability of attaining the receptor than the hydrophobic cyclodiene insecticides. Conversely, the more internal sites would not be easily available to the polar agents due to lipophilic penetration barriers including the integument and and the neural sheaths surrounding the insect CNS. The taste receptor of an insect is essentially a "naked" dendrite surrounded by a fluid-filled sinus within a cuticular shell on the exterior mouth and tarsal parts of the insect (Figure 7). These sensitive chemosensory receptors are the insect's window to its chemosphere, and may provide a rapid screen for susceptibilities in pest populations to GABA-acting insecticides of moderate polarity.

## Involvement of Detoxification Enzymes in Antifeedant Toxicity to WCR.

A STLA pool of predominantly the epoxides (**1**,**2**, and **4**) was used in the studies that follow. Adult WCR were topically treated with established inhibitors of xenobiotic-metabolizing enzymes (*10*) at sublethal dosages 2 hr prior to injection of 10 μg or less of STLAs. Inhibitors screened included piperonyl butoxide (for cytochrome P450-dependent monooxygenases), diethylmaleate (glutathione transferase), DEF (carboxylesterases) and 1,2-epoxy-3,3,3-trichloropropane oxide (epoxide hydrolase). A weak synergism of the 24 hr acute toxicity of STLAs occurred with diethylmaleate and piperonyl butoxide, suggesting a glutathione transferase and cytochrome P450 involvement in STLA detoxification.

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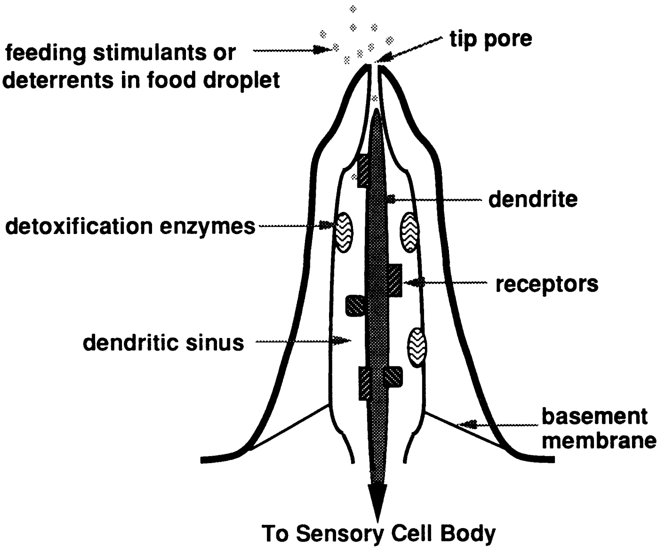

Figure 7. Gross structure adapted from ref. *9* of a contact chemosensory sensillum on galea of insect maxilla. Putative presence of detoxification enzymes is included.

## Conclusions

Cross-resistance in insects between plant allelochemicals and synthetic insecticides have been implicated mostly based on allelochemical induction or inhibition of xenobiotic-metabolizing enzymes (*4, 5, 99,100*), but its absence has also been evident (*101, 102*). However, peripheral chemoperception that drives fixed-behavior patterns may be also modulated by fortuitous encounters with unnatural insecticides. For example, sublethal amounts of permethrin can impair sex pheromone communication in the pink bollworm (*103*). The question remains if these chemical encounters may force selection of either sensory or more internal resistances to natural dietary and xenobiotic toxicants.

To our knowledge, no study has addressed the involvement of gustatory chemosensory neuroreceptors in possible target-site-based cross-resistance between natural and synthetic insecticides. Chlorinated cyclodiene susceptibility in WCR, a major corn pest, is modified by adult feeding on alternative host plants of the family Asteraceae (*15*). Epoxysesquiterpene lactone antifeedants isolated from sunflower exhibit picrotoxinin-like $GABA_A$-gated chloride ionophore neurotoxicities in corn rootworm (*17*). GABA receptor-directed chemistry including picrotoxinin, bicuculline and related isoquinoline alkaloids, and other MDPs including piperonyl butoxide and piperine are potent antifeedants for WCR. Among cyclodiene-resistant and susceptible strains of *Diabrotica*, a correlation between levels of cyclodiene epoxide resistance and

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antifeedant activities for bicuculline, picrotoxinin and other GABA receptor ligands is emerging. Goodness of fit by molecular modeling to a picrotoxinin or a bicuculline binding site indicates that a GABA-like receptor mediates antifeedant and internal effects of sunflower on WCR, and thereby increases WCR susceptibility to cyclodienes. *Diabrotica* may by behavioral avoidance of GABA antagonists in their marginal host plants such as sunflower delay the decay of cyclodiene resistance. These sensitive taste receptors on sensillar dendrites which are virtually outside the insect body may provide a rapid screen for susceptibilities to GABA-acting insecticides in pest populations. Compelling is the possibility that genes of both chemosensory and central GABA receptors may be coordinately expressed.

The $GABA_A$ receptor is particularly attractive as a target for insect control since invertebrates in contrast to vertebrates have numerous peripheral in addition to central nerve sites that are regulated by this inhibitory neurotransmitter. This cosmopolitan receptor for numerous drugs and toxicants in vertebrates may be the basis in *Diabrotica* of both cucurbitacin action and the antifeedant behavior of argophyllins and other terpenoid epoxides including azadirachtin and citrus limonoids. Net chemical interactions at peripheral gustatory receptors are determining the insect's commitment to feed. For example, corn rootworm will feed on what are otherwise antifeedants or neurotoxicants (this study) as well as on insecticidal baits such as Nemesis (MicroFlo Company) developed by R. L Metcalf and associates (*104*) if the feeding stimulatory cucurbitacins are present. The latter terpenoids although "sweet" to the *Diabrotica* are "bitter" and toxic to other insects and animals. This strategy to control pest insects via "stomach" poisons that elude rejection by sensitive taste receptors is akin to a mandate given in reference to control of human behavior found in Revelation 10:9 of the Bible.

**"Take it, and eat it; and it will make your
stomach bitter, but in your mouth it will be sweet as honey"**

If GABA-dependent chloride ionophores mediate sensillar transduction of insect gustatory behavior, chemically disabling receptors responsible for feeding will lead to insect starvation and death. Novel compounds that impair feeding behavior in insecticide resistant WCR may provide an environmentally-safe, low-chemical-input strategy for corn production.

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